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Establishment of Induced Pluripotent Stem Cells From Rat Fibroblasts

Inaugural-Dissertation

zur Erlangung der Doktorwürde der
Vetsuisse-Fakultät Universität Zürich

vorgelegt von

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Zürich 2009

Für meine Eltern

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Abstract

In vitro, embryonic stem (ES) cells, which are derived from the inner cell mass of mammalian blastocysts, have the ability to remain in an undifferentiated state for an infinite period of time while maintaining pluripotency and the capability to differentiate into cells of all three germ layers. Generation of pluripotent cells can also be achieved by epigenetic reprogramming of somatic cells either by nuclear cloning, fusion with ES cells or lately by retrovirus-mediated introduction of defined factors (Oct-3/4, Sox2, Klf4 and c-Myc), which enables the generation of induced pluripotent stem (iPS) cells that are indistinguishable from ES cells.

In the mouse ES cells are widely and successfully used to generate gene targeted animals but in the rat this was until very recently not possible because of the absence of germline competent rat ES cells. Therefore, the newly developed iPS cell technique provides a feasible approach to establish pluripotent stem cells from rat fibroblasts, cells that could be further used for the generation of targeted rats.

The aim of this thesis was to reprogram rat embryonic fibroblasts to rat iPS cells by introducing defined factors. In addition, in this work we tried to optimize the iPS technique to reprogram gene-targeted rat knock-out fibroblasts to iPS cells which can be used to generate gene-targeted rats.

Embryonic rat fibroblasts were reprogrammed using either four (Oct-3/4, Sox2, Klf4 and c-Myc) or three factors (Oct-3/4, Sox2 and Klf4). The established rat iPS cells were characterized by karyotyping and testing the expression of typical markers for pluripotency: alkaline phosphatase (AP), Oct-3/4 and SSEA-1. In a next step the differentiation potential of the generated rat iPS cells was examined by embryoid body formation, *in vitro* differentiation into specific cell types and inducing teratoma formation after injection into nude mice. Finally the ability to generate chimeric rats was tested by performing blastocyst injections.

The established rat iPS cell, regardless if reprogrammed with four or three factors were positive for the expression of AP, Oct-3/4 and SSEA-1, formed embryoid bodies, differentiated *in vitro* to cell types representing the mesoderm and the ectoderm and were able to form teratomas consisting of derivatives from all three germ layers in nude mice. After blastocyst injection chimeric rats could be generated, the percentage of chimerism of the newborn rats still has to be determined.

The gene-targeted rat fibroblasts were reprogrammed using the three factors Oct-3/4, Sox2 and Klf4. The resulting iPS cells have not been further characterized and were frozen after the reprogramming process due to time restraints.

Zusammenfassung

Embryonale Stammzellen leiten sich aus der inneren Zellmasse von Säugetierblastozysten ab. Sie zeichnen sich durch die Fähigkeit aus, *in vitro* für unbegrenzte Zeit ihren undifferenzierten Zustand beibehalten zu können; gleichzeitig bleiben sie pluripotent und sind in der Lage, Zellen aller drei Keimblätter zu bilden. Es gibt verschieden Möglichkeiten, Körperzellen epigenetisch zu embryonalen Stammzellen umzuprogrammieren. Man kann ihren Zellkerninhalt in Eizellen übertragen oder sie mit embryonalen Stammzellen verschmelzen. Seit kurzer Zeit ist es auch möglich, aus Körperzellen von Mensch oder Maus sogenannte induzierte pluripotente Stammzellen (iPS) herzustellen, indem man mit Hilfe von Retroviren bestimmte Faktoren (Oct-3/4, Sox2, Klf4 und c-Myc) in die Zelle einschleust. iPS sind von embryonalen Stammzellen nicht zu unterscheiden.

Embryonale Stammzellen der Maus werden häufig und mit Erfolg verwendet, um genetisch zielgerichtet veränderte Tiere zu erzeugen. Für die Ratte allerdings war dies bis vor kurzem nicht möglich, da keine zur Keimbahnübertragung befähigten embryonalen Stammzellen der Ratte zur Verfügung standen. Die neu entwickelte iPS-Technologie stellt deshalb einen machbaren Ansatz zur Etablierung pluripotenter Stammzellen aus Fibroblasten der Ratte dar; diese Zellen könnten dann zur Herstellung genetisch veränderter Ratten verwendet werden.

Ziel dieser Arbeit war die Reprogrammierung embryonaler Ratten-Fibroblasten zu Ratten-iPS durch das Einführen definierter Faktoren in die Fibroblasten. Zusätzlich haben wir versucht, durch Verbesserung der iPS-Technologie, zielgerichtet genetisch modifizierte Rattenfibroblasten, sogenannte Knock-out (KO) Zellen, zu iPS umzuprogrammieren, welche dann zur Erzeugung von KO Ratten verwendet werden können.

Zum Reprogrammieren der embryonalen Ratten-Fibroblasten wurden entweder vier (Oct-3/4, Sox2, Klf4 und c-Myc) oder drei (Oct-3/4, Sox2 und Klf4) Faktoren verwendet. Die so erzeugten Ratten-iPS wurden anschliessend durch die Bestimmung des Karyotyps charakterisiert, ausserdem wurden sie auf die Expression typischer Pluripotenzmarker hin untersucht: Alkalische Phosphatase, Oct-3/4 und SSEA-1. In einem weiterführenden Schritt wurde das Differenzierungspotential der erzeugten Zellen überprüft: Die Ausbildung embryoähnlicher Zellaggregate, sogenannter „embryoid bodies“, die *in vitro* Differenzierung zu bestimmten Zelltypen und die Tumorbildung nach Injektion der iPS in Nacktmäuse.

Unabhängig davon, ob mit drei oder mit vier Faktoren reprogrammiert wurde, waren die erzeugten Ratten-iPS positiv für die Expression von alkalischer Phosphatase, Oct-3/4 und SSEA-1; sie bildeten embryoid bodies aus, differenzierten sich *in vitro* zu Zell-Typen aus Mesoderm und Ektoderm und sie bildeten in Nacktmäusen Tumore, die aus Abkömmlingen aller drei Keimblätter bestanden. Die

Blastozysten-Injektion führte zur Geburt chimärer Jungtiere, allerdings muss noch der Grad der Ausprägung bestimmt werden.
Die Ratten-KO-Fibroblasten wurden mit den drei Faktoren Oct-3/4, Sox2 und Klf4 reprogrammiert. Die daraus entstandenen iPS wurden anschliessend aus Zeitgründen vorerst eingefroren.

Abbreviations

2i	Two inhibitors
3i	Three inhibitors
AP	Alkaline Phosphatase
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
bp	Base pair
cDNA	Complementary DNA
c-Myc	Myelocytomatosis oncogene
DAPI	4'-6-Diamidino-2-phenylindole
D-MEM	Dulbecco modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
dsDNA	Double-stranded DNA
EB	Embryoid bodies
EC cells	Embryonic carcinoma cells
EG cells	Embryonic germ cells
EGFP	Enhanced green fluorescent protein
ENU	N-ethyl-N-nitrosourea
Env	Envelope
ES cells	Embryonic stem cells
EtOH	Ethanol
FBS	Fetal bovine serum
Fbx15	F-box-containing protein 15
FCS	Fetal calf serum
FGF	Fibroblast growth factor
Fig.	Figure
G418	Geneticin
Gag	Group specific antigen
GFAP	Glial fibrillary acidic protein
GSK 3	Glycogen synthase kinase-3
h	Hour
HE-staining	Hematoxylin and eosin staining
hiPS cells	Human iPS cells
HMG	High mobility group
HR	Homologous recombination
HSV-tk	Herpes simplex virus thymidine kinase
ICM	Inner cell mass
Ig	Immunoglobulin
iPS cells	Induced pluripotent stem cells
iPS-3 cells	Three-factor induced iPS cells (Klf4, Sox2, Oct3/4)

iPS-4 cells	Four-factor induced iPS cells (Klf4, Sox2, Oct3/4, c-Myc)
IRES	Internal ribosomal entry site
Klf4	Kruppel-like factor 4
KO	Knock-out
LB medium	Luria Bertani medium
LIF	Leukemia inhibitory factor
MEF	Mouse embryonic fibroblast
MEK	Mitogen-activated protein kinase
min	Minutes
NBT	Nitro blue tetrazolium chloride
Neo	Neomycin phosphotransferase
NT	Nuclear transfer
o/n	Over night
Oct-3/4	Octamer-binding transcription factor-3/4
OD	Optical density
PBS	Dulbecco's phosphate buffered saline
PBT	Phosphate buffer saline + 1% Tween 20
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
Plat-E cells	Platinum-E cells
Pol	Polymerase
REF	Rat embryonic fibroblast
rES cells	Rat embryonic stem cells
rES-like cells	Rat embryonic stem-like cells
rH	Relative humidity
riPS cells	Rat iPS cells
RNA	Ribonucleic acid
rpm	Rounds per minute
RT	Room temperature
RT-PCR	Real time polymerase chain reaction
s	Seconds
SCID	Severe combined immunodeficiency
SMA	Smooth muscle actin
Sox2	SRY-related HMG-box gene 2
SSEA-1	Stage-specific embryonic antigen-1
TS cells	Trophoblast stem cells
VPA	Valproic acid
XEN	Extraembryonic endoderm cells

A. Introduction

1. Pluripotent stem cells

By definition stem cells have the ability to remain in an undifferentiated state for an infinite period, but maintain the capability to differentiate into various cell types (Boiani and Scholer, 2005; Ralston and Rossant, 2005; Wobus and Boheler, 2005). Stem cells can be found in embryos, fetuses and adults.

Embryonic stem (ES) cells give rise to the cells of all three germ layers – the mesoderm, the endoderm and the ectoderm – and these cells further differentiate into all the specialised cells of the adult organism.

Adult stem cells can be isolated from multiple organs and tissues. These cells keep the potential to differentiate into cells belonging to different germ layer derived lineages (Rietze et al., 2001; Stemple and Anderson, 1992; Toma et al., 2001).

1.1. Pluripotent murine embryonic stem cells

Murine embryonic stem (mES) cells are derived from the epiblast, a region of the inner cell mass (ICM) of the blastocysts at embryonic day 3.5 (E3.5) (Brook and Gardner, 1997; Evans and Kaufman, 1981; Martin, 1981). These cells can be indefinitely cultured, under certain conditions, without losing their pluripotency and their stable karyotype.

Two other cell types besides the mES cells are derived from the early mouse embryo: the trophoblast stem (TS) cells and the extraembryonic endoderm (XEN) cells (Evans and Kaufman, 1981; Martin, 1981; Tanaka et al., 1998). TS cells descend from the trophectoderm lineage of the blastocyst. XEN cells derive from the primitive endoderm (Fig. 1) (Kunath et al., 2005; Tanaka et al., 1998).

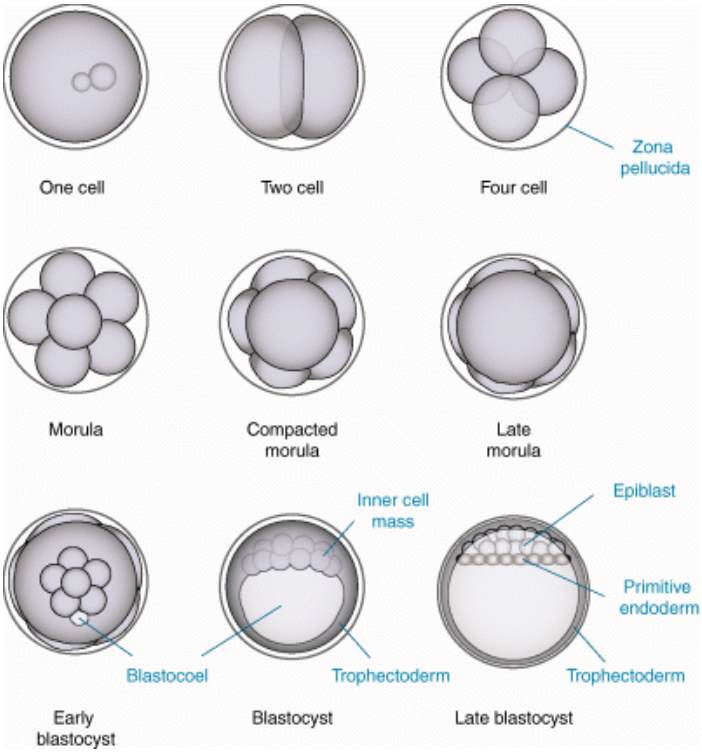

Fig. 1: Development of the mouse blastocyst. Three successive rounds of cell division follow the fertilization of the oocyte and lead to the formation of the morula. The outer layer of the late morula forms an epithelium called the trophectoderm. Around this time, the blastocoel forms and moves the ICM to one end of the blastocyst. During late blastocyst stages, just prior to hatching from the zona pellucida, cells of the ICM become organized into epiblast and primitive endoderm populations. From (Ralston and Rossant, 2005).

1.1.1. Characteristics of pluripotent murine embryonic stem cells

In vitro, pluripotent cells display a typical morphology. The colonies have a round shape, exhibit shiny boarders and grow in compact multilayers. Furthermore, the cells have a high nucleus-to-cytoplasm ratio.

Undifferentiated mES cells express factors, which are commonly used as pluripotency markers: For example stage-specific embryonic antigen-1 (SSEA-1), a specific cell surface antigen (Solter and Knowles, 1978), and octamer-binding transcription factor–3/4 (Oct-3/4), a germ line-specific transcription factor (Scholer et al., 1989). Furthermore, they have active alkaline phosphatase (AP) (Wobus et al., 1984) and telomerase activity (Thomson et al., 1998).

In vitro pluripotent cells are able to form embryo-like aggregates called “embryoid bodies” (EB) and they differentiate after induction with specific factors into various cell types of all three germ layers (Fig. 2) (Wobus et al., 1984).

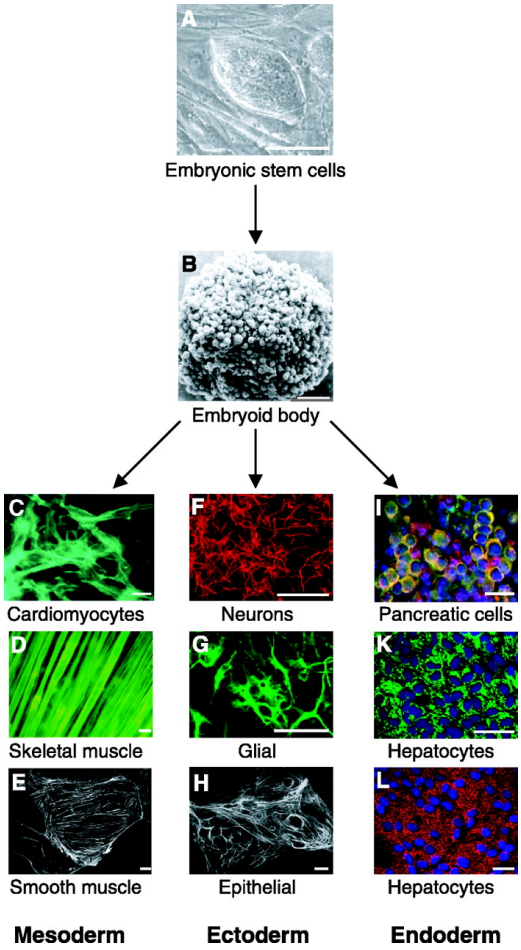

Fig. 2: *In vitro* differentiation of ES cells. Undifferentiated mouse ES cells (A) develop *in vitro* via three-dimensional aggregates (embryoid body, B) into differentiated cell types of all three primary germ layers. Differentiated cell types labelled with tissue-specific antibodies are shown: C: cardiomyocytes (titin Z-band epitope). D: skeletal muscle (titin Z-band epitope). E: smooth muscle (smooth muscle-actin). F: neuronal (βIII tubulin). G: glial (glial fibrillary acidic protein, GFAP). H: epithelial cells (cytokeratin 8). I: pancreatic endocrine cells [insulin (red), C-peptide (green), insulin and C-peptide co-labelling (yellow)]. K and L: hepatocytes (K, albumin; L, 1-antitrypsin). Bars = 0.5 µm (H), 20 µm (I), 25 µm (C, D, E), 30 µm (K, L), 50 µm (B, G), and 100 µm (A, F). From (Wobus and Boheler, 2005).

mES cells which were cultivated *in vitro* lead to teratoma formation when injected subcutaneously into the flank of SCID (severe combined immunodeficiency disorder) mice. The teratomas consist of different cell types representing all three germ layers (Wobus et al., 1984).

In vivo, mES cells participate in the development of an embryo after morula aggregation or reintroduction into an unrelated blastocyst (Wobus et al., 1984). Due to their epiblast origin, mES cells rarely contribute to the extraembryonic endoderm and the trophoblast (Beddington and Robertson, 1989).

Teratoma formation and the ability to reintegrate into embryogenesis, represent the *in vivo* differentiation capacity of mES cells.

1.2. Rat embryonic stem-like cells

Rat embryonic stem-like (rES-like) cells are derived from the ICM of a blastocyst at embryonic day 4.5 (Demers et al., 2007). rES-like cells exhibit growth characteristics similar to mouse ES cells, including a high nucleus-to-cytoplasma ratio and growth as tightly packed aggregates. Both were morphological characteristics of undifferentiated cells (Wobus and Boheler, 2005). rES-like cells express AP, SSEA-1 and Oct-3/4, even after transfection, selection, cryopreservation and thawing (Demers et al., 2007). AP, SSEA-1 and Oct-3/4 are typical markers of undifferentiated embryonic stem cells. In suspension, rES-like cells form cystic structures, known as EB, which develop several cell layers reminiscent of the early embryo. Injected into SCID mice, rES-like cells form teratomas containing differentiated cells and tissues belonging to all three germ layers (Iannaccone et al., 1994). After blastocyst injection of rES-like cells and embryo transfer, the cells are able to contribute to functional extraembryonic tissues during development (Demers et al., 2007). However, rES-like cells are not able to contribute to developing embryonic tissue or colonize the germ line. Both of these features are essential for demonstrating the true pluripotent character of ES cell lines (Demers et al., 2007).

1.2.1. Rat embryonic stem cells

Ueda *et al.* (2008) claimed to have established rat embryonic stem (rES) cells which are capable of producing chimeric rats. The cells show the same morphology in culture as pluripotent mouse embryonic stem cells. Long term stable culture conditions for these cells have been established. When cultivated, they maintain a normal karyotype. rES cells are positive for SSEA-1 and Oct-3/4, but negative for AP activity. The cells published by Ueda *et al.* are able to form EB and to differentiate into a wide variety of cell types of the three germ layers. *In vivo*, rES cells are capable of forming teratoma after transplantation into SCID mice but the tumors contained only cells from two germ layers – the endoderm and the mesoderm. The cells are capable of producing chimeric rats after blastocyst injection, but were not able to reconstitute the germ line. Further investigations have to be done, firstly to see if these results are reproducible in independent repeated experiments and secondly to test if it is possible to generate a germline chimera, which is a criterion for considering these cells pluripotent.

Recently, Buehr *et al*. (2008) have reported the successful generation of rat ES cells. These rES were cultivated in medium containing inhibitors of signals, which initiate loss of pluripotency and entry into differentiation. Therefore they combined either three inhibitors (3i), targeting FGF (fibroblast growth factor) receptor, mitogen-activated protein kinase (MEK) and glycogen synthase kinase-3 (GSK3), or two inhibitors (2i), targeting MEK and GSK3, with LIF (leukemia inhibitory factor) and incubated the rES cells under these conditions. The established 3i/LIF and 2i/LIF rES cells express key markers of pluripotency, e.g. Nanog and Oct-3/4, lead to teratoma formation after injection into SCID

mice and are able to generate rat chimeras, furthermore the 2i/LIF cells are able to colonise the germ line.

2. Nuclear reprogramming and induced pluripotency in somatic cells

ES cells, because of their pluripotent state, have theoretically the capacity to differentiate into any cell type of the body, and offer therefore the possibility to be used as a source for medical and therapeutic indications, e.g. treatment of degenerative diseases.

Authentic ES cell lines are able to colonize the germ line, exist only in mice and chicken (Keefer et al., 2007; Wobus and Boheler, 2005). Hence methods of inducing nuclear reprogramming and pluripotency in somatic cells open a door to generate ES-like cells in other species than mouse and chicken. Furthermore the generation of pluripotent cells directly from individual somatic cells offers a possible way to circumvent the problem of tissue rejection following transplantation.

In the following sections the three most widely used methods for inducing nuclear reprogramming are described.

2.1. Reprogramming by nuclear transfer

Nuclear transfer (NT) involves transplantation of the nucleus from an adult somatic cell into an enucleated, unfertilized oocyte. The resulted diploid cell is then activated to initiate the first cell cycle and undergoes reprogramming of its genetic material. The cell begins then to divide, leading to the formation of a blastocyst *in vitro*. The resulting blastocyst is then either implanted into the uterus of a pseudopregnant female to ensure full-term development - this process is called reproductive cloning - or the blastocyst is used for ICM extraction for the derivation and propagation of an ES cell line. In this case the process is known as therapeutic cloning.

NT in mammals was first developed in the sheep, with the cloning of Dolly (Wilmut et al., 1997). The number of species cloned after Dolly, including e.g. cow (Kato et al., 1998), mouse (Wakayama et al., 1998), pig (Polejaeva et al., 2000), cat (Shin et al., 2002), rat (Zhou et al., 2003), horse (Galli et al., 2003) and dog (Lee et al., 2005), demonstrated the potential capacity of this technique to reprogram somatic nuclei to pluripotent nuclei and even to generate a whole animal.

2.2. Reprogramming by fusion with ES cells or with cell extracts

Fusion of somatic cell nuclei with ES, embryonic germ (EG) or embryonic carcinoma (EC) cells leads also to reprogramming of its genetic material. This strategy was first published by Harris *et al.* (1965). They showed that the fusion of HeLa cells - a cell line derived from cervical cancer cells from Henrietta Lacks - with Sendai virus was able to activate erythrocytes (Harris, 1965; Harris and Watkins, 1965; Harris et al., 1965). Subsequently Miller *et al.* (1976) demonstrated that thymocytes became pluripotent after fusion with mouse EC cells. Nuclear reprogramming of somatic cells was

later obtained by electrofusion with mouse embryonic germ (EG) cells (Tada et al., 1997) and mouse ES cells (Tada et al., 2001).

These results lead to the assumption that ES cells contain factors which induce pluripotency in somatic cells. It is still controversial whether these factors are localised in the nucleus (Do and Scholer, 2004) or in the cytoplasm (Strelchenko et al., 2006) of ES cells. However, pluripotent cells generated by fusion of somatic cells and ES cells contain both somatic cells and ES cell-derived chromosomes. Matsumura *et al.* (2007) developed a system to circumvent this problem by removing whole chromosomes from tetraploid cells.

As an alternative to reprogramming by fusion with complete ES cells, Taranger *et al.* (2005) attempted to reprogram somatic cells using ES cell extracts. They used extracts from the nucleus and cytoplasm, assuming that these extracts contained the necessary regulatory components.

To date this strategy is only partially successful (Taranger et al., 2005) and further investigations are necessary to improve the reprogramming degree caused by the use of cell extracts.

2.3. Reprogramming with defined factors

The fact that somatic cells can be reprogrammed by transferring their nuclear contents into oocytes or by fusion with ES cells indicates that unfertilized eggs and ES cells contain reprogramming factors. These factors are able to confer totipotency or pluripotency to somatic cells.

Takahashi *et al.* (2006) hypothesized that reprogramming factors largely overlap with those maintaining pluripotency in ES cells. They identified four factors, Oct-3/4, Sox2 (SRY-related HMG-box gene 2), Klf4 (Kruppel-like factor 4) and c-Myc (myelocytomatosis oncogene), which are able and necessary to induce reprogramming in somatic cells after introduction by retroviral transduction. The generated pluripotent cells are called induced pluripotent stem (iPS) cells.

Reprogramming by defined factors is more precisely described in chapter 3.

2.4. Summary

In Table 1 and Fig. 3 the three reprogramming methods are summarized.

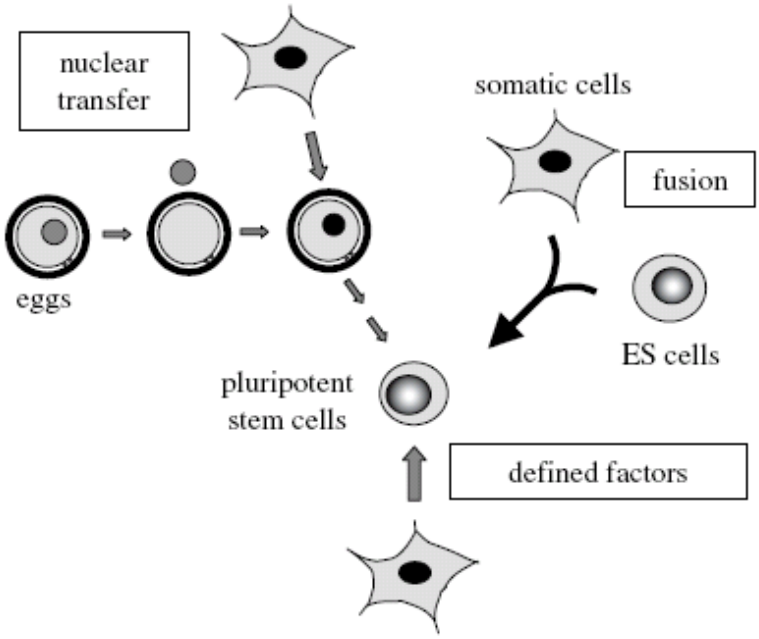

Fig. 3: An overview of the three methods of inducing pluripotency in somatic cells (Yamanaka, 2008). Nuclear transfer: transferring a somatic cell nucleus into an enucleated egg. Fusion: fusing somatic cells with ES cells. Defined factors: introducing defined factors into somatic cells.

	Nuclear transfer	**Fusion with ES cells**	**Stable expression of defined factors**
Description	Introduction of somatic cell nucleus into an enucleated oocyte	Hybrids of differentiated and pluripotent cells	Exogenous expression of Oct-3/4, Sox2, Klf4 and c-Myc
Use of embryos /donor oocytes	Yes	(Yes)	No
Chromosome content	Normal diploid	Tetraploid	Retroviral integration
Restriction	Limited by availability of oocytes and low cloning efficiency	Additional set of chromosomes	Side effects through reactivation of transgenes

Table 1: Summary of the three methods used for inducing pluripotency in somatic cells.

3. Reprogramming by defined factors

Reprogramming of somatic cells can either be induced by nuclear transfer, fusion with ES cells or induction with defined factors, which is the most recent method.

Reprogramming with defined factors is the method used in my project. It is therefore described more precisely in the following sections.

3.1. Induction of pluripotent stem cells from somatic cells by defined factors

Somatic cells can be reprogrammed by transferring their nuclear contents into enucleated oocytes (Wilmut et al., 1997) or by fusion with ES cells (Tada et al., 2001). This indicates that unfertilized eggs or ES cells contain factors that are able to confer pluripotency to somatic cells. Takahashi and Yamanaka (2006) (Takahashi and Yamanaka, 2006) hypothesised that a factor that plays a pivotal role in the maintenance of ES cell identity could also play an important role in the induction of pluripotency in somatic cells.

They selected 24 genes, which were known to be involved in the maintenance of pluripotency in early embryos and ES cells. To evaluate these 24 genes, they developed an assay system in which the induction of the pluripotent state in the reprogrammed somatic cells could be detected as a resistance to G418 (Geneticin), an aminoglycoside antibiotic. To do so, they inserted a βgeo cassette, containing a neomycin resistance gene, into the mouse Fbx15 (F-box protein 15) gene by homologous recombination (HR) (Fig. 4). Fbx15 is dispensable for the maintenance of pluripotency, but is specifically expressed in mouse ES cells and early embryos (Tokuzawa et al., 2003).

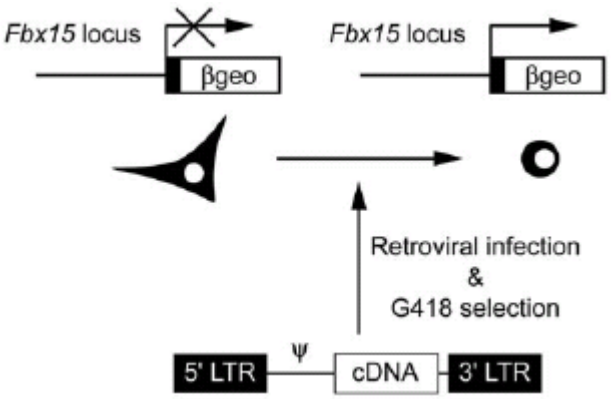

Fig. 4: System to test candidate factors. Retroviral infection leads to a pluripotent state of the somatic cells. Due to the pluripotent state the Fbx15 promoter gets activated driving transcription of the βgeo cassette and conferring G418 resistance to the reprogrammed cells. From (Takahashi and Yamanaka, 2006).

All 24 candidate genes were introduced into mouse embryonic fibroblasts (MEFs) by retroviral transduction (Morita et al., 2000) and the cells were then cultured in ES cell medium containing G418. G418-resistant colonies were generated and some of them exhibited morphology similar to ES cells, including a round shape, large nucleoli and scant cytoplasm (Fig. 5A).

Reverse transcription PCR (RT-PCR) analysis showed that these iPS colonies expressed ES cell markers, e.g. Oct-3/4 and Nanog. However the iPS colonies showed different DNA methylation patterns compared to ES cells (Fig. 6A). To determine which of the 24 candidates were critical to induce reprogramming in MEFs, they examined the effect of the withdrawal of one individual factor from the pool of the 24 genes. They therefore infected MEFs twenty-four times always using 24 factors minus 1 individual factor. G418-resistant colonies were not formed when either Oct-3/4 (factor 14), Sox2 (factor 15) or Klf4 (factor 20) was removed. Removal of c-Myc (factor 22) resulted in colonies with a flatter, non ES cell-like morphology (Fig. 5B). The introduction of these four genes resulted in the formation of G418-resistant colonies. This data indicated that Oct-3/4, Sox2, Klf4 and c-Myc were necessary for the generation of iPS cells from MEFs.

RT-PCR analysis revealed that the iPS cells generated by the introduction of the four genes (iPS-4) expressed the majority of the marker genes for pluripotency. Furthermore the colonies were positive for AP, SSEA-1 and showed a high telomerase activity. They examined the pluripotency of iPS-4 cells *in vivo* by teratoma formation after injection into nude mice and *in vitro* by EB formation (Fig. 5D). Histological examination of the resulted teratomas revealed that iPS-4 cells differentiated into all three germ layers (Fig. 5C).

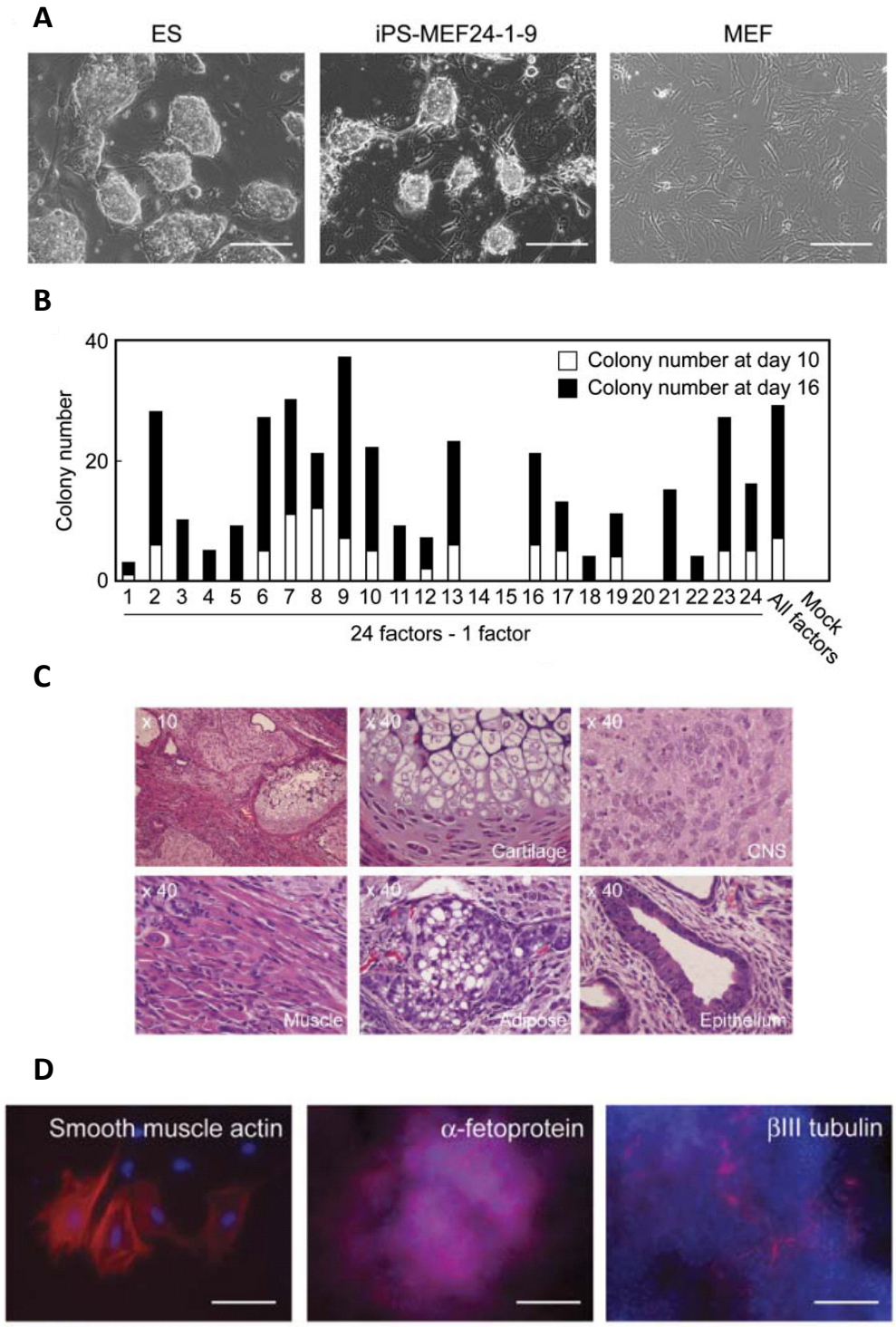

Fig. 5: **A)** Morphology of ES cells, iPS cell generated by 24 factors and MEFs. Scale bars = 200µm. **B)** Effect of the removal of individual factors from the pool of 24 transduced factors on the formation of G418-resistant colonies. The transduced MEFs were selected with G418 for 10 days or 16 days. **C)** Various tissues from all three germ layers, mesodern (cartilage, muscle), endoderm (columnar epithelium) and ectoderm (squamous epithelium, neural tissue (CNS)) present in teratomas derived from iPS-4 cells. **D)** Immunostaining confirming the *in vitro* differentiation into all three germ layers, mesoderm (smooth muscle actin), endoderm (α-fetoprotein) and ectoderm (βIII tubulin). Scale bars = 100µm. Adapted from (Takahashi and Yamanaka, 2006).

They also introduced the four factors into tail-tip fibroblasts to examine whether these four factors, Oct-3/4, Sox2, Klf4 and c-Myc, were able to induce pluripotent cells from adult mouse fibroblasts. The resulting iPS cells expressed most of the ES cells marker genes, e.g. Nanog, Oct-3/4 and Sox2 (using RT-PCR with primers that amplified endogenous but not transgenic transcripts), were positive for AP and were able to form teratomas containing tissues of all three germ layers. Furthermore, iPS cells contributed to mouse embryonic development after injection into blastocysts.

In summary it can be said that these iPS cells proved the feasibility to generate pluripotent stem cells directly from fibroblast cultures by defined factors. However, the generated iPS cells were not able to colonize the germ line, the frequency of iPS cell derivation was low and the global gene-expression patterns and DNA methylation status were not identical to ES cells.

In later experiments, selection for Nanog or Oct-3/4 expression instead of Fbx15 expression resulted in germ line-competent iPS cells with increased ES cell-like gene expression and DNA methylation patterns (Fig. 6B) (Okita et al., 2007; Wernig et al., 2007).

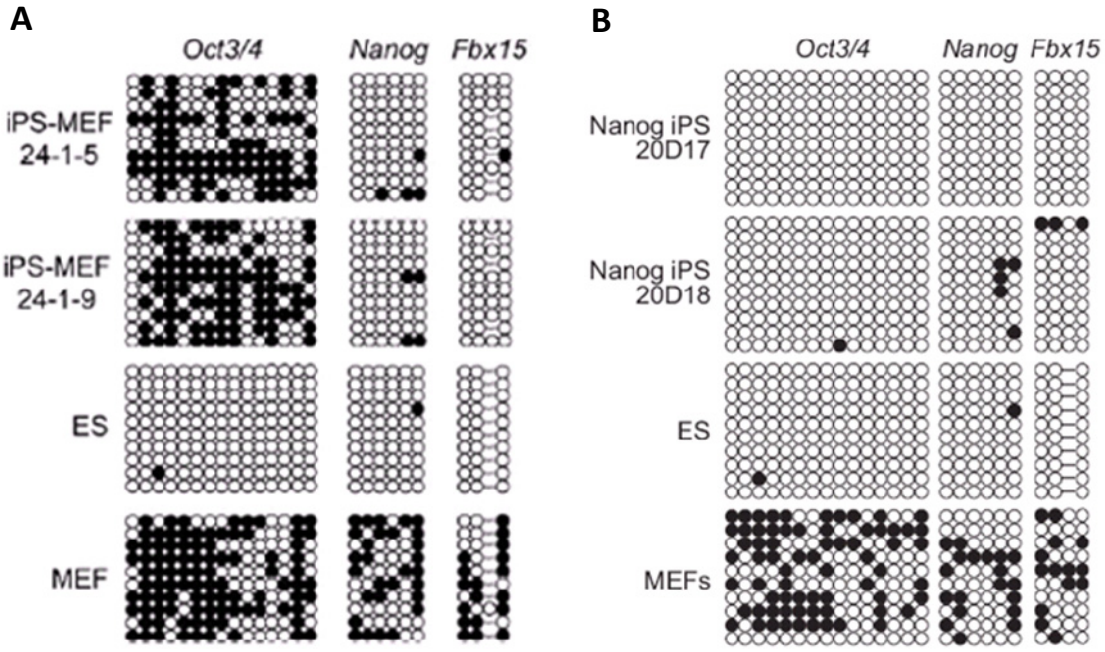

Fig. 6: **A)** Bisulfite genomic sequencing of the promoter regions of Oct-3/4, Nanog, and Fbx15 in iPS cells selected for Fbx15 expression, ES cells, and MEFs. **B)** Bisulfite genomic sequencing of the promoter regions of Oct-3/4, Nanog, and Fbx15 in iPS cells selected for Nanog expression, ES cells, and MEFs. Open circles indicate demethylation, while closed circles indicate methylation. Adapted from (Okita et al., 2007) and (Takahashi and Yamanaka, 2006).

One of the negative effects of reprogramming with 4 factors is the observation of Okita *et al.* (2007) that the reactivation of c-Myc leads to tumor formation in F_1 (first filial generation) mice derived from the Nanog-iPS cell line.

Nakagawa *et al.* (2007) and Wernig *et al.* (2008) demonstrated that fibroblasts can be reprogrammed to a pluripotent state by Oct-3/4, Sox2 and Klf4 in the absence of c-Myc. This offers the possibility to circumvent the problem of tumor formation caused by c-Myc-retrovirus reactivation. The omission of c-Myc resulted in a less efficient but more specific induction of MEFs to murine iPS cells (Nakagawa et al., 2008).

In parallel, human iPS cells were generated from human somatic cells using the same factors, Oct-3/4, Sox2, Klf4 and c-Myc or using a modified quartet of factors, Oct-3/4, Sox2, Nanog and Lin28 (Takahashi et al., 2007; Yu et al., 2007). Also human iPS cell can be generated without c-Myc, which is important for clinical applications (Nakagawa et al., 2008).

Later it was shown, that valproic acid (VPA), a histone deacetylase inhibitor, enables reprogramming of human fibroblasts with only two factors, Oct-3/4 and Sox2. This is an important step toward an improved safety in therapeutically application of reprogrammed cells, caused by the absence of the two oncogenes c-Myc and Klf4 (Huangfu et al., 2008b).

Brambrink *et al*. (2008) devised a reprogramming strategy using doxycycline-inducible lentiviral vectors, which allows a controlled expression of all four transgenes. Furthermore, they showed that the virally transduced cDNA (complementary DNA) needed to be expressed for at least 12 days in order to generate murine iPS cells.

Recently it was demonstrated, that the generation of mouse iPS cells is possible without viral vectors. Okita *et al.* (2008) established a method, in which repeated transfection of a single plasmid containing Oct-3/4, Sox2 and Klf4, together with a c-Myc expressing plasmid is sufficient to generate iPS cells without evidence of plasmid integration. This could be an important step toward future application of iPS cells in regenerative medicine and other clinical usage.

3.2. Factors

The induction of reprogramming in somatic cells was achieved by introducing the four factors, Oct-3/4, Sox2, Klf4 and c-Myc (Takahashi and Yamanaka, 2006) or Oct-3/4, Sox2, Nanog and Lin28 (Yu et al., 2007), usually used for the reprogramming of human somatic cells.

In our project we reprogrammed somatic cells using the four factors Oct-3/4, Sox2, Klf4 and c-Myc. Therefore, these four factors are described in the following sections.

As preliminary remark it has to be said that the importance of the two factors Oct-3/4 and Sox2 for pluripotency is well determined (Chambers and Smith, 2004; Ivanova et al., 2006; Masui et al., 2007), but the role of the two oncogenes c-Myc and Klf4 in reprogramming is less clear.

3.2.1. Oct-3/4

Oct-3/4, also known as Oct3, Oct4 or Pou5F1, is a member of the POU specific-domain containing family of transcriptional factors. This factor is able to act with various other transcription factors and is associated with a large number of target genes implicated in maintenance of pluripotency.

The expression of Oct-3/4 in mice is restricted to early embryos, more precisely in the cleavage stage blastomeres, the ICM and the epiblast of the egg cylinder, and to primordial germ cells at gastrulation. Postnatally the expression of Oct-3/4 is limited to growing oocytes and spermatogonia (Scholer et al., 1989). Oct-3/4-deficient embryos die *in utero* at pre-implantation developmental stages (Nichols et al., 1998). *In vitro* cultivation of the ICM derived from these Oct-3/4-deficient embryos leads to differentiation into trophoblast, whereas the overexpression of this protein causes differentiation into primitive endoderm and mesoderm (Niwa et al., 2000).

Summarized, Oct-3/4 plays an important role both in the derivation and maintenance of ES cells (Pesce et al., 1998).

3.2.2. Sox2

Sox2 is an abbreviation for sex determining region Y (SRY) box 2. Sox2 is a SRY-related HMG (high mobility group)-box family transcription factors that is expressed in ES cells, early embryos, germ cells and adult neural stem cells (Koopman et al., 2004). In contrast to Oct-3/4, Sox2 is also required in the extraembryonic ectoderm. However it does not appear to play a role in primitive endoderm formation (Avilion et al., 2003). Sox2-deficient embryos die around implantation stage caused by a failure in epiblast development.

Summarized, Sox2 plays a key role in the self-renewal and pluripotency of ES cells.

3.2.3. Klf4

Kruppel-like factor 4 (Klf4) is a zinc finger-type transcription factor that is expressed in a wide variety of tissues, e.g. the intestine, and in undifferentiated mouse ES cells (Yamanaka, 2008). It can function either as a tumor suppressor or an oncogene due to differences in cell context and expression patterns of other genes (Evans and Liu, 2008). Klf4 cooperates with Oct-3/4 and Sox2 to activate the Lefty1 core promoter (Nakatake et al., 2006) and Klf4 represses also p53 (Rowland et al., 2005), a negative regulator of Nanog during ES cell differentiation (Lin et al., 2005).

An important point in the context of reprogramming by defined factors is that the repression of p53 might function as an inhibitor of Myc-induced apoptosis (Zindy et al., 1998).

3.2.4. c-Myc

c-Myc is a helix-loop-helix/leucine zipper transcription factor and acts as an oncogene. c-Myc takes part in a broad variety of cellular functions, its target genes participate in cell cyle, survival and protein synthesis (Dang et al., 2006) and c-Myc is thought to modify chromatin structure (Knoepfler

et al., 2006). c-Myc itself is regulated by STAT3 and is also a common target of the Wnt signaling pathway.

In the context of generating iPS cells, c-Myc may induce global histone acetylation (Fernandez et al., 2003), thus allowing Oct-3/4 and Sox2 to bind to their specific target loci. Besides it may compensate anti-proliferative effects of Klf4.

3.3. Problems and their possible solutions

Reprogramming somatic cells to pluripotent stem cells by defined factors is the latest reprogramming method. During the last three years this technique passed through different states of development including revelation of problems and their possible solutions, which leads to a continuous progress in this strategy.

In the following sections some of these problems and their solutions are described.

3.3.1. Selection by the activation of Oct-3/4 or Nanog

iPS cells established by the selection of Fbx15 expression and subsequently G418-resistance exhibited similar characteristics to ES cells, but they show differences with regards to gene expression and DNA methylation patterns, and fail to produce adult chimeras (Takahashi and Yamanaka, 2006). Therefore Wernig *et al.* (2007) described a similar process by which generated iPS cells more closely resemble ES cells at the molecular level (Fig. 6). Not only do iPS cells exhibit a very similar morphology and proliferation pattern to ES cells, but they also express pluripotency markers, and show *in vitro* differentiation potential. Furthermore these cells can form viable chimeras and contribute to germ line. Their generated MEFs carried a neomycin-resistance marker inserted by HR into either the endogenous Oct-3/4 or Nanog locus. MEFs were then infected with retroviral vectors to induce expression of the four factors and cultivated in medium containing G418. The expression of the endogenous Oct-3/4 or Nanog gene, as a marker for induced pluripotency, led to G418-resistent colonies.

This strategy enabled the generation of germ line competent iPS cells with gene expression and DNA methylation patterns similar to the one of ES cells.

3.3.1.1. Selection by the morphology

Meissner *et al.* (2007) demonstrated that reprogrammed pluripotent cells can be isolated from somatic cells solely based upon morphological criteria, without drug selection.

3.3.2. c-Myc is dispensable for reprogramming

Beside the function of c-Myc as an important modulator of cellular functions, c-Myc acts as an oncogene. In the context of reprogramming, reactivation of the c-Myc retrovirus increases tumorigenicity in the chimeras and progeny mice (Okita et al., 2007). Wernig *et al.* (2007) and

Nakagawa *et al.* (2007) demonstrated the generation of induced pluripotent stem cells with Oct-3/4, Sox2 and Klf4 retroviruses in the absence of the c-Myc retrovirus. iPS cells generated by the introduction of only 3 factors (iPS-3) exhibit ES cell characteristic. They are positive for AP activity and immunofluorescent staining confirmed the expression of the pluripotency markers Nanog and SSEA-1. Furthermore iPS-3 cell led to the formation of teratomas containing various cell types representing all three germ layers and generated chimeras after injection into blastocysts. Nevertheless the reprogramming process without c-Myc was delayed and less efficient, but led to a more specific induction of MEFs to iPS cells.

Nakagawa *et al*. (2007) also succeeded in generating human iPS cells from adult dermal fibroblasts without c-Myc.

Summarized, reprogramming somatic cells to a pluripotent state by Oct-3/4, Sox2 and Klf4 in the absence of c-Myc represents an important step forward in the production of less-tumorigenic iPS cells.

3.3.3. Klf4 and c-Myc are dispensable for reprogramming

c-Myc and Klf4 are well known oncogenes and the integration of their viral transgenes in generation of pluripotent stem cells leads to concerns with regard to safety for a possible clinical application.

Huangfu *et al*. (2008) demonstrated that VPA, a histone deacetylase inhibitor, enables reprogramming of human fibroblasts with only two factors, Oct-3/4 and Sox2. Furthermore, VPA increases the efficiency of reprogramming human fibroblasts to pluripotent stem cells. The two-factor induced human iPS cells (hiPS-2) were morphologically similar to human ES cells. This was confirmed by positive staining for AP and immunofluorescence staining for pluripotency markers, e.g. Nanog, Sox2 and Oct-3/4. RT-PCR analysis showed that expression levels of pluripotency marker genes in hiPS-2 were similar to those of human ES cells. *In vitro* differentiation potential of hiPS-2 cells was confirmed by their ability to form EB and subsequent differentiation in derivatives of the three germ layers. Furthermore the hiPS-2 cells formed teratomas after injection into SCID mice. The teratomas contained tissues originating from the three germ layers.

In summary, in the presence of VPA, Oct-3/4 and Sox2 is sufficient for reprogramming human fibroblasts to a pluripotent state.

3.3.4. Stability of iPS cells generated from brain-derived neural stem cells

Silva and Barrandon *et al.* (2008) showed, that mouse brain-derived neural stem (NS) cells reprogram faster than other cell types. However these reprogrammed cells remain in a state close to full pluripotency, as demonstrated by the lack of a stable expression of endogenous Oct-3/4 and Nanog, the lack of epigenetic erasure of X chromosome silencing in female cells and the inability to generate chimeras. The authors developed a system, which blocks the signals that initiate the loss of

pluripotency and the entry into differentiation in the embryo. Their protocol consists in combining two inhibitors (2i) of MEK signalling and GSK3 with the self-renewal cytokine LIF. The 2i/LIF condition allowed the partly converted neural stem cells to complete the transition efficiently, as demonstrated by stable up-regulation of Oct-3/4 and Nanog, reactivation of the X chromosome, transgene silencing and competence to form germline chimeras. Furthermore using 2i/LIF Oct-3/4 and Sox2 is sufficient to convert NS cells into chimera-forming iPS cells.

This system leads in NS cells to a fast reprogramming paired with a high incidence of conversion.

This data may help to establish conditions for a faster reprogramming and a more stable cultivation of reprogrammed fibroblasts.

3.3.5. Reprogramming without using retroviruses

A major risk and limitation of reprogramming is the use of potentially harmful genome-integrating viruses, which can lead to tumour formation due to spontaneous reactivation of the viral transgenes.

Stadtfeld *et al.* (2008) generated iPS cells by using a non-integrating adenoviruses transiently expressing Oct-3/4, Sox2, Klf4 and c-Myc. iPS cells generated in that manner showed DNA demethylation patterns similar to ES cells, express endogenous pluripotency genes, form teratomas and contribute to multiple tissues, including the germ line, in chimeric mice. The efficiency of deriving iPS cells is lower than that obtained with integrating viruses. This is probably due to the fact that many cells do not maintain the viral expression long enough to induce complete reprogramming (Brambrink et al., 2008; Stadtfeld et al., 2008a). It has to be tested, whether the low efficiency of adenoviral reprogramming can be increased by the use of chemical compounds as has been reported for retroviral reprogramming (Huangfu et al., 2008a). This work showed that stable integration is not required for *in vitro* reprogramming.

Other works performed by Okita *et al.* (2008) pushed the iPS technology further towards safe clinical applications, because their protocol does not require viral vectors for reprogramming. They placed the three factors Oct-3/4, Klf4 and Sox2 into a plasmid vector and in addition, they constructed another plasmid to express c-Myc. Repeated transfection of these two plasmids into MEFs resulted in iPS cells without evidence of plasmid integration. To confirm their pluripotency, the iPS cells were injected into nude mice. All clones gave rise to teratomas containing different cell types including cells derived from all three germ layers. After injection the iPS cells into blastocyst, adult chimeras were generated. It remains to be tested, whether virus-free iPS cells are germ line-competent and whether they can be generated from adult somatic cells.

Nevertheless the production of iPS cells without viral vectors fulfils a critical safety concern for potential use of iPS cells in regenerative medicine.

4. Modification of the genome

The manipulation of the genome offers an informative and direct way to understand the function of genes and to associate these functions to pathophysiological processes. Therefore the generation of animal models with gain or loss of gene function is an important field in research.

4.1. Modification of the rat and mouse genome

Nowadays the mouse and the laboratory rat belong to the central experimental animal models in several fields of biomedical research.

The mouse is by far the most studied model organism due to the establishment of germ line-competent ES cells, which can be modified by gene-targeting via transfection with modified homologous DNA. The modified ES cells give then rise to a germline chimera after injection into a blastocyst and transfer to a foster mother. Via HR in ES cells, a great variety of genetically modified mice, especially genetically deficient mice, have been generated in the last years. This technique of gene-targeting is one of the most powerful tools to investigate gene regulation and function *in vivo.*

On the other hand the rat is one of the most studied animal model organism for human diseases such as cardiovascular diseases, infectious diseases, cancer and is used as a model for aging, autoimmunity, transplantation biology, industrial toxicology, pharmacology, behavioural and addiction studies (Gill et al., 1989). The size of the rat is regarded as advantageous over the mouse model.

In 2004 the rat joined the mouse and human as the third mammal whose complete genome sequence has been determined (Gibbs et al., 2004). The analysis of the genome sequences showed an enrichment of genes involved in immunity, metabolic detoxification and reproduction and conservation of genes involved in human diseases (Gibbs et al., 2004). Furthermore the rat has a higher genetic diversity compared to the mouse (Canzian, 1997), which leads to a more accurate representation of human pathologies due to the possibility to overcome species-specific factors.

Until today it was not possible to generate knock-out rats by inducing HR in ES cells. An important step in this direction was recently achieved by the group of Austin Smith with the generation of the first rES cell (see also section 1.2.1.). In this publication Buehr *et al.* (2008) showed that 2i/LIF cells could be stably electroporated, an indication that would be also possible to introduce DNA constructs for HR into rES cells.

4.2. Gene-targeting via ES cells

4.2.1. The mechanism of homologous recombination

In 1984 Bradley *et al.* demonstrated the capacity of murine ES cells to colonize the germ line of mouse chimeras produced by blastocyst injection. This opened the possibility to generate targeted mutations in the mouse germ line. Three years later the first targeted mutation could be introduced into ES cells in culture by HR (Thomas and Capecchi, 1987). The first genetically modified mouse produced by using gene-targeting via HR in ES-cells was finally born in 1989 (Thompson et al., 1989).

A targeting vector containing the requested mutation and a marker flanked by sequences homologous to the genomic target is used to introduce a desired mutation into the germ line of mice. The vector is introduced into an ES cell line by transfection. The introduced vector pairs with its homologues chromosomal DNA sequence and the mutated sequence replaces the wild-type sequence by HR. These altered ES cells are injected into wild-type blastocysts and transferred into recipient mice. The resulting chimera may be identified by coat colour or reporter genes, e.g. green fluorescent protein (GFP) gene (Gagneten et al., 1997).

To verify the HR and eliminate ES cells, which underwent random insertion, positive selection for purposeful integration for the targeting vector can be combined with an additional marker, which selects against ES cells carrying randomly integrated vectors (called negative selection). In this case the targeting vector contains the positive marker within the region of homology and the negative marker outside the homologous DNA. The most commonly-used positive marker is the prokaryotic neomycin phosphotransferase (neo) gene which leads to resistance against neomycin and its analogue G418. The herpes simplex virus thymidine kinase gene (HSV-tk) is the current marker used for negative selection. It converts nucleoside analogues such as ganciclovir into metabolites, which are toxic for cells. This strategy allows enriching ES cell clones with a targeted integration event, because the terminal part (containing the HSV-tk) should be cleaved-off after HR (Fig. 7).

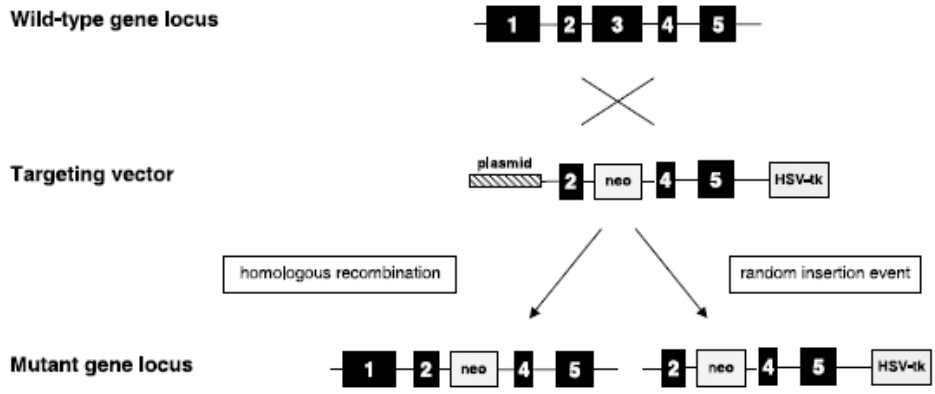

Fig. 7: Positive-negative selection. The targeting vector contains the positive selection marker neo and the negative selection marker HSV-tk. HR leads to resistance to G418 and ganciclovir. Random integration confers resistance to G418, but induces sensitivity to ganciclovir. From (Prelle et al., 2002).

4.2.2. Homologous recombination in somatic cells and nuclear transfer

It has been shown that gene-targeting in somatic cells and subsequent nuclear-transfer is possible in several species such as cow (Sendai et al., 2006), sheep (McCreath et al., 2000), and pig (Lai et al., 2002). First the genome of a somatic cell has to be genetically modified and then the nucleus of this cell is used as donor and injected into an enucleated oocyte. The resulting embryo is then implanted into the uterus of a pseudopregnant female and the reconstructed embryo can develop to an offspring. However several limitations remain, e.g. low homologous efficiency, mixed population of targeted and non-targeted cells after selection and expansion. For the rat this strategy is not available, however it has been possible to clone the rat by nuclear transfer (see section 2.1.) (Zhou et al., 2003).

In our laboratory, Oliver Sterthaus (2007) attempted to generate gene-targeted rat by NT during his PhD-Thesis. In this project the Neurotrypsin locus was targeted in rat embryonic fibroblast by HR. The nucleus of these cells was then used as donor for the injection into enucleated oocytes. After activation and subsequent transfer to the oviduct of a foster mother, the reconstructed embryos should develop to a chimeric rat. Unfortunately during this project it was only possible to generate knock-out (KO) fibroblasts by HR, but no chimeric rat could be established because of technical troubles with NT.

4.3. Reprogramming and knock-out rats

Reprogramming of somatic cells, as described in the chapter 3, offers a new ES cell independent technique to generate knock-out animals, which we hope to be able to successfully adapt to the rat. Two possible ways to generate transgenic rats by reprogramming are conceivable. The first one consists in inducing HR in somatic cells and then reprogram these cells to rat iPS (riPS) cells. The second one consists in reprogramming of somatic cells to riPS cells and then to induce HR in the riPS cells.

The genetically modified riPS cells are then injected into a blastocyst in order to obtain a knock-out germline chimera, which could generate knock-out or knock-in offspring. This technique resembles the method of HR in somatic cells and subsequent nuclear transfer. However, the main advantage of the reprogramming of somatic cells is the fact that the use of oocyte to induce pluripotency in somatic cells is not anymore necessary. Nevertheless, blastocysts are still necessary to generate chimeric animals. For sure, further investigation and experiments have to be done to prove and establish this technique.

5. Aim of the work

The rat is an important laboratory animal, e.g. due to its size and its higher genetic diversity compared to the mouse. However, despite many attempts to do so, it still has not been possible to establish rat ES cell cultures. Therefore the generation of ES cells by reprogramming rat fibroblasts with these four factors could be a new powerful method to establish pluripotent rat stem cells and maybe subsequent generation of genetically modified rats.

The following experiments are planed:

- Reprogramming of rat embryonic fibroblasts to pluripotent stem cells by introducing either four factors (Oct-3/4. Sox2, Klf4 and c-Myc) or three factors (Oct-3/4, Sox2 and Klf4) using retroviruses
- Expansion of the resulting riPS cells
- Karyotype analysis of the riPS cells in order to preclude chromosomal abnormalities
- PCR analysis to amplify the introduced transgenes
- Alkaline phosphatase staining and immunohistochemistry of these riPS cells in order to check their pluripotency
- EB formation, *in vitro* differentiation and teratoma formation in order to verify their differentiation potential
- Electroporation of riPS cells with an vector encoding EGFP (enhanced green fluorescent protein)
- Blastocyst injection in order to generate chimeric rat, another examination of the pluripotent state

B. Materials

1. Chemicals, biochemicals and enzymes

Bacteriological peptone, SIGMA
Yeast extract, SIGMA
Sodium chloride 100mM, Fluka
Select Agar, SIGMA
Ampicillin, 50mg/ml, SIGMA
Trypsin-EDTA solution 10x, SIGMA
Mitomycin C, SIGMA
Dulbecco's phosphate buffered saline, SIGMA
QIAfilter™ Plasmid Maxi Kit, QIAGEN
Fugene 6, Transfection Reagent, Roche
Polybrene Infection / Transfection Reagent, Millipore
pEGFP-N3 vector, Clontech
Kanamycin, 10mg/ml, SIGMA
SacI, 10U/µl, Promega
MULTI-CORE™ Buffer, Promega
Ethanol, Fluka
NaOAc, 3M Sodium acetate buffer solution pH5.2, SIGMA
Demecolcine, SIGMA
Methanol p.a., Merck
Acetic acid , Merck
Giemsa , SIGMA
Proteinase K, Roche
Phenol :Chloroform:Isoamylalcohol 25:24:1, Fluka
PCR buffer 10x, QIAGEN
$MgCl_2$ 25mM, QIAGEN
dNTP mix, 10mM each, QIAGEN
Taq Polymerase, 5u/µl, QIAGEN
Q-solution 5x, QIAGEN
Mass Ruler™ DNA Ladder Low Range, Fermentas
Paraformaldehyde, Fluka

4-Nitro blue tetrazolium chloride, Roche
5-Bromo-4-chloro-3-indolyl-phosphate, X-phosphate, 760 994, Roche
Ethylenediaminetetraacetic acid (EDTA), pH 8.0, Fluka
Triton® x-100 SigmaUltra, SIGMA
Horse Serum, GIBCO
DAPI (4'-6-Diamidin-2'-phenylindoldihydrochlorid), Roche
Rabbit anti Oct-3/4 polyclonal IgG, 200µg/ml, Santa Cruz Biotechnology
Mouse anti SSEA-1 monoclonal IgM, 1mg/ml, Chemicon
Goat anti Oct-3/4 polyclonal IgG, 200µg/ml, Santa Cruz Biotechnology
Alexa Fluor® 594 goat anti rabbit IgG, 2mg/ml, Invitrogen
Alexa Fluor® 488 goat anti mouse IgG, 2mg/ml, Invitrogen
Alexa Fluor® 594 goat anti mouse IgG, 2mg/ml, Invitrogen
βIII tubulin, SIGMA
Glial fibrillary acidic protein, DAKO
Smooth muscle actin, SIGMA

2. Definitions and preliminary remarks

- The methods are generally described. If nothing else noted means that the same protocols for the three and four factors cells were used.
- In the following pages the term fibroblasts is always used to define embryonic fibroblasts that are not mitotically inactivated. As feeders are always defined mitomycin C inactivated fibroblasts.
- All media were stored at 4°C and preheated to 37°C before use.

3. Medium

3.1. Bacteria

Luria Bertani (LB) medium

10g/l Bacteriological Peptone

5g/l Yeast Extract

10g/l NaCl

Autoclave at 121°C for 20min.

LB medium with Agar

10g/l Bacteriological Peptone

5g/l Yeast Extract

10g/l NaCl

15g/l Select Agar

Autoclave at 121°C for 20min. Let cool down and add 50mg/ml Ampicillin. Mix the medium on a magnetic stirrer. Prepare bacteriological Petri dishes.

3.2. Platinum-E (Plat-E) cells

Derived from 293T cell line

Plat-E cells contain an env-IRES-puro cassette and a gag-pol-IRES-cassette.

Plat-E cell medium (FP-medium)

D-MEM 1x, GIBCO

10% Fetal Bovine Serum, heat-inactivated, GIBCO

L-Glutamin, Penicillin, Streptomycin (10.000 U/ml penicillin G sodium; 10.000µg/ml streptomycin sulphate; 29.2mg/ml L-glutamine; 10mM sodium citrate in 0.14% NaCl), GIBCO

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

Plat-E cell medium (FP-medium antibiotics)

D-MEM 1x, GIBCO

10% Fetal Bovine Serum, heat-inactivated, GIBCO

L-Glutamin, Penicillin, Streptomycin (10.000 U/ml penicillin G sodium; 10.000µg/ml streptomycin sulphate; 29.2mg/ml L-glutamine; 10mM sodium citrate in 0.14% NaCl), GIBCO

Puromycin (1:10’000) 10mg/ml, GIBCO

Blasticidin S (1:1’000) 10mg/ml, GIBCO

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

3.3. Rat fibroblast

Derived from Wistar RCC

Fibroblast medium (rFP-medium)

D-MEM 1x, GIBCO

10% Fetal Bovine Serum, heat-inactivated, GIBCO

Penicillin, Streptomycin (10.000 U/ml penicillin G sodium, 10.000µg/ml streptomycin in 0.9% NaCl), Sigma

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

3.4. SNL fibroblast

Obtained from Dr. Allan Bradley (Sanger Institute).

Derived from a STO cell line (mouse)

SNL 76/7 STO cells contain a neomycin-resistant cassette and expressing LIF.

SNL-medium

D-MEM 1x, GIBCO

7% Fetal Bovine Serum, heat-inactivated, GIBCO

L-Glutamin, Penicillin, Streptomycin (10.000 U/ml penicillin G sodium; 10.000µg/ml streptomycin sulphate; 29.2mg/ml L-glutamine; 10mM sodium citrate in 0.14% NaCl), GIBCO

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

3.5. riPS-cells

iPS-medium

D-MEM 1x, GIBCO

15% Fetal Bovine Serum, heat-inactivated, GIBCO

L-Glutamin, Penicillin, Streptomycin (10.000 U/ml penicillin G sodium; 10.000µg/ml streptomycin sulphate; 29.2mg/ml L-glutamine; 10mM sodium citrate in 0.14% NaCl), GIBCO

ß-Mercaptoethanol 50 mM, GIBCO

MEM Non Essential Amino Acids 100X, GIBCO

The solution was sterilized by filtration through 0.22µm filters and stored at 4°C.

Supplemented with:

10^7U/ml, 10.000X ESGRO murine LIF, Chemicon Int.

Selection medium with G418 (iPS-medium G418)

iPS-medium

Geneticin (G418) (50mg/ml), GIBCO

iPS-cells freezing medium

Fetal Bovine Serum, heat-inactivated, GIBCO

10% DMSO (Dimethyl sulphoxide), SIGMA

3.6. Blastocyst injection

M2 Medium, without Penicillin, Streptomycin, Lactic Acid and Sodium Bicarbonate, SIGMA

M16 Medium, with Sodium Bicarbonate and Lactic Acid, without Penicillin and Streptomycin, SIGMA

KSOM Medium, with Amino Acids and D-Glucose, Millipore

Injection-medium

iPS-medium

2% HEPES 1M, GIBCO

4. Culture dishes

100mm Petri dishes, Falcon

24/96 Multidishes, Corning

35/60/100mm plates, Corning

150mm plates, TPP

T25, T75 flasks, Corning

5. Material

15ml/50ml tubes, GREINER

CryoTube™ vials, Nunc™

Filter bottles (0.2µm pores), Corning

Gene Pulser® Cuvette, 0.4cm, BIORAD

Serological pipettes, Sarstedt or Falcon

UVette® 30-2000µl, Eppendorf

6. Equipment

Centrifuge (Megafuge 2. ORS), Heraeus

Centrifuge 5810R, Eppendorf

Centrifuge 5418, Eppendorf

Minifuge 5415R, Eppendorf

Gel documentation system, Alpha Innotech

Gene Pulser® II, BIORAD

MicroPorator, Digital Bio

PCR machine: T1 Thermocycler, Biometra

Spectrophotometer: Specgene, Techne

Thermo mixer, Eppendorf

Incubator Cytoperm2, Heraeus

Microscope: Nikon TMS / Nikon Eclipse E600 / Nikon SMZ 800

Fluorescence microscope: Axiovert 40 CFL, ZEISS

Sterile bench: VSE 2000-120, SKAN

7. Software

Clone Manager®

AxioVision Rel. 4.6

C. Methods

1. Rat feeders / SNL feeders

Mitotically inactivated fibroblasts are used as a basal layer for riPS-cells and secret factors, provide extracellular matrix and cellular contacts to maintain the cells in an undifferentiated and pluripotent state.

1.1. Production of Rat fibroblasts

Rat fibroblasts were derived from embryonic day 14.5 (E14.5) Wistar-RCC fetuses.

The fetuses were dissected from the uterus. The head and the organs were removed and the rest of the fetuses was homogenised using 1x trypsin.

Finally the cells were resuspended in rFP-medium and transferred to 150mm dishes ($5x10^6$ cells/150mm). They were incubated at 37°C, 5% CO_2 and 95% relative humidity (37°C, 5% CO_2, 95% rH) until the cells became confluent. At this point the cells were either expanded or frozen as passage 0.

1.2. Expansion of Rat fibroblasts

Rat feeders are quite difficult to expand, because they almost do no adhere to the dish. Due to this the fibroblasts were plated in 1 gelatinized 150mm plate instead of 4 gelatinized 150mm plates after thawing, which is the standard protocol used for the expansion of mouse fibroblasts. Furthermore, they were only expanded as far as passage 1.

1.3. Expansion of SNL-fibroblasts

At day 1 a vial of SNL-feeders was thawed and plated in four 150mm plates, using 20ml of SNL-medium for each plate. During the next 5 days the cells were cultivated without changing the medium. At day 6 the plates were confluent and the cells were mitotically inactivated.

1.4. Inactivation of Rat fibroblasts and SNL fibroblasts

Rat fibroblasts were inactivated at passage 0 or passage 1. For the inactivation of SNL-fibroblast any passage can be used. The inactivation is performed with mitomycin-C, a cytostatic antibiotic. Before starting with inactivation, fresh mitomycin medium was prepared: 2ml PBS were injected into the mitomycin bottle to yield 1mg/ml and added to 200ml rFP-medium. The medium was removed and 7ml mitomycin containing medium were added. The cells were incubated for 2hs at 37°C. After the incubation the medium was aspirated from the plates and the cells were washed three times with 15ml PBS. Afterwards 7ml 1xtrypsin was added to the cells and incubated at 37°C for 3min. The cells were then resuspended in SNL-medium and distributed to two 50ml tubes. Aliquots of 10µl were taken to determine the amount of cells. The suspension was centrifuged for 4min at 210g. The

pellets were resuspended in 4-6ml freezing medium, depending to the cell density, and aliquoted in 4-6 crytotubes containing 7 million cells each. The tubes were stored for one to five days at -80°C and then transferred in the liquid nitrogen tank.

2. Reprogramming

2.1. Maxi Preparation

The vectors were kindly offered by S. Yamanaka. We received 4 clones of pre-transformed DH5α bacteria, frozen in glycerol. Each of the four clones contained a vector encoding one of the 4 murine factors necessary for reprogramming. The bacteria were plated on agar plates containing 50mg/ml ampicillin and the plates were incubated overnight at 37°C.

The next day 100ml LB medium containing 100µl of a 50mg/ml stock ampicillin were inoculated with a colony of each plate. The colonies were picked with a sterile pipette tip. The solution was incubated overnight at 37°C shaking at 210g. The day after the 100ml cultures were centrifuged at 2057g for 15min and the supernatant was decanted. The DNA isolation steps were achieved with the QIAfilter™ Plasmid Maxi Kit according to the QIAfilter Plasmid Maxi® protocol. At the end the DNA was eluted in 500µl water and stored in Eppendorf tubes at -20°C.

For quantifying the concentration of DNA, 5µl DNA from Maxi preparations were diluted in 495µl water (1:100) and the absorption of UV light was measured with a spectrophotometer at 260/280nm. DNA has an absorption maximum of 260nm. An absorbance of 1 OD (Optical Density) is equivalent to 50µg/ml dsDNA at 260nm.

2.2. Reprogramming

The following protocol describes reprogramming with four factors, when cells were reprogrammed with only three factors only medium containing viruses with Oct-3/4, Sox2 and Klf4 was used. In this case, the protocol is the same except that only three 100mm dishes with Plat-E cells were necessary. Three factors cells grow slower than cells reprogrammed by four factors, so the colonies became big enough to be picked at around day 20.

Day 1: Two vials of frozen Plat-E cells were thawed, suspended in 40ml of FP-medium containing puromycin (1:10'000) and blasticidin S (1:1'000) (FP-medium antibiotic) and then transferred to four gelatin-coated 100mm dishes. The cells were incubated at 37°C, 5% CO_2, 95% rH.

Day 2: The medium was replaced with FP-medium antibiotic.

Day 3: The Plat-E cells have reached a confluence of about 80-90% and they were ready for transfection. 1.2ml of DMEM was distributed into four Eppendorf tubes and 27µl of Fugene 6

transfection reagent was added, mixed carefully by finger tapping and incubated for 5min at room temperature. After incubation, 9µg of pMXs plasmid DNA (encoding Oct-3/4, Sox2, Klf4 and c-Myc) was delivered drop-by-drop to the Fugene 6-DMEM solution, mixed gently by finger tapping and incubated for 15min at room temperature. Then the DNA-Fugene 6 complex was finally added dropwise into the four Plat-E dishes and these were incubated overnight (o/n) at 37°C, 5% CO_2, 95% rH. The FP-medium used in this period contains neither puromycin nor blasticidin S (FP-medium). A gelatin-coated 100mm dish was prepared with rat feeders and 10ml FP-medium.

Day 4: The transfection reagent-containing medium was replaced by 10ml of fresh FP-medium and the cells were returned to the incubator.

Day 5: The virus-containing medium was collected from the Plat-E dishes using sterile disposable syringes, filtered through 0.45µm pore size cellulose acetate filters and transferred into four 15ml tubes (one tube for each factor). Afterwards 5µl of 8mg/ml polybrene solution was added into the filtrated medium and mixed carefully by pipetting up and down. A mixture of equal parts of the medium containing either Oct-3/4-, Sox2-, Klf4- or c-Myc-retroviruses was made. $1.5x10^6$ rat fibroblasts were added to 10ml of the polybrene/virus containing medium and this solution was used to replace the FP-medium in the 100mm dish prepared at day 3. The cells were incubated at 37°C, 5% CO_2, 95% rH.

Day 6 and Day 7: After 24hs and 48hs the medium from the dish containing the fibroblasts was aspirated and fresh FP-medium was added.

Day 8: The FP-medium was removed and 10ml of iPS-medium was added.

Following days: The iPS-medium was changed every day until the colonies became big enough to be picked. The colonies became large enough to be picked around day 17.

3. Picking of reprogrammed cell clones

A 96well plate was prepared with 30µl PBS per well. The iPS-medium was not removed for picking, because the cells detach when being incubated to long in PBS. Single colonies were picked by using a 10µl pipette tip and were added to 30µl of PBS in the 96well plate. The colonies were trypsinised with 30µl 2x trypsin and were incubated for 5min in the incubator. Afterwards 140µl iPS-medium was added to each well and the colonies were resuspended using a 1000µl pipette.

The SNL-medium was removed from the 24well plate with feeders, which have been prepared about 2hs before. 300µl iPS-medium was added to each well in the 24well plate. 200µl of the resuspended colonies were transferred to the 24well plate and incubated at 37°C, 5% CO_2, 95% rH.
When the cells reached a confluence of about 70%, they were split 1:1 in a 35mm dish and then routinely passaged when confluent to 60mm plates. At this point the different clones were frozen and stored in liquid nitrogen.

4. Cultivation of riPS-cells

4.1. Thawing of riPS-cells

Frozen riPS-cells were thawed in 37°C water bath, transferred in 9ml SNL-medium and centrifuged at 210g for 4min. The supernatant was removed and the pellet was resuspended in the required amount of iPS-medium, depending on the diameter of the used dishes. The medium was removed from the feeder cells, which had been prepared before thawing the riPS-cells. Afterwards the resuspended riPS-cells were transferred on the feeder cells and cultivated at 37°C, 5% CO_2, 95% rH.

4.2. Splitting riPS-cells

Splitting is necessary, when the cells reach a confluence of about 70%. The medium was removed, the cells were washed once with PBS and 1ml of 1x trypsin (for 60mm dishes) was added. After a 5min long incubation at 37°C, the cells had detached. They were then resuspended in 4ml SNL-medium by pipetting up and down and transferred in a 15ml tube containing 5ml of SNL-medium. The solution was centrifuged for 4min at 210g. The pellet was resuspended in iPS-medium and the cells were transferred 1:2 to 1:10 (depending on growth rate) in feeder dishes. The cells were cultivated at 37°C, 5% CO_2, 95% rH.

	96well	24well	35mm	60mm	100mm	T75
Feeder	$2x10^6$	$2.4x10^6$	$0.4x10^6$	$1x10^6$	$3x10^6$	$3x10^6$
iPS-medium LIF	200µl/well	500µl/well	2ml	5ml	10ml	15ml
1x Trypsin	30µl	200µl	500µl	1ml	3ml	3ml

Table 1: Amount of feeders, medium and trypsin pro culture plate type.

4.3. Freezing riPS-cells

The iPS-medium was removed and the dish was washed once with PBS. The required amount of 1x trypsin was added and the cells were incubated for 5min at 37°C. Afterwards the cells were resuspended in SNL-medium (1.5ml for 35mm dishes, 4ml for 60mm dishes) by pipetting up and down and added to 8ml (for 35mm dishes) or 5ml (for 60mm dishes) SNL-medium. The cell suspension was centrifuged for 4min at 210g and the supernatant was removed. The pellet was

resuspended in the freezing medium, using 1ml freezing medium per cryotube and aliquoted in cryotubes. The cells were immediately stored one to five days at -80°C and then transferred in the liquid nitrogen tank.

5. Electroporation pEGFP-N3

The riPS-cells were electroporated with EGFP to see, if the reprogrammed cells can still be electroporated after reprogramming and also have green cells for injection into blastocyst for generating chimeric rats. The vector pEGFP-N3 was used for the electroporation.

5.1. Maxi Preparation EGFP

Three Erlenmeyer flasks each with 100ml LB medium containing 20µl of a 10mg/ml stock kanamycin were inoculated with bacteria containing pEGFP-N3 out of a glycerol stock. The solution was incubated overnight at 37°C shaking at 210rpm.

The day after the 300ml cultures were centrifuged at 2057g for 15min and the supernatant was decanted. The pellets was resuspended with 10ml P1 Buffer and transferred in one 50ml tube. The following steps were achieved with the QIAfilter™ Plasmid Maxi Kit according to the QIAfilter Plasmid Maxi® protocol. At the end the DNA was eluted in 500µl water and stored in Eppendorf tubes at -20°C.

For quantifying the concentration of DNA, 5µl DNA from Maxi preparations was diluted in 495µl water (1:100) and the absorption of UV light was measured with a spectrophotometer at 260/280nm. The concentration amounts to 2.3µg/µl.

5.2. Linearization of pEGFP-N3

The pEGFP-N3 vector was linearised before being electroporated, so that it would stably integrate into the genomic DNA of the riPS-cells. For this purpose the vector was digested with SacI.

Restriction digestion reaction:

20µl pEGFP-N3 vector (40µg)

10µl SacI (10U/µl)

10µl 10x buffer MC

60µl water

The reaction was incubated overnight at 37°C. The following day the performance of restriction was controlled by gel electrophoresis. The linearised vector should be 4700bp long. Afterwards the DNA was precipitated by adding to the restriction solution 2.5 Vol. 100% ethanol and 1/30 Vol. NaOAc pH 5.2. The solution was mixed and incubated for at least 1h at -80°C. Then the tube was centrifuged at 4°C for 15min at 13200g and the pellet was resuspended in 40µl water. The linear vector was stored at -20°C.

5.3. Electroporation

For electroporation a 70% confluent 60mm riPS containing dish was used, containing 6 million cells. At first six 60mm dishes were prepared with SNL feeders, which were transfected with a G418-resistance cassette. Each dish was prepared with 5ml iPS-medium.

Three factors

The cells were washed with 5ml PBS and trypsinised during 5min in the incubator. Afterwards the cells were resuspended in 9ml SNL-medium and centrifuged at 210g for 5min. The pellet was resuspended in 10ml PBS and in order to determine the cell density 10µl suspension was removed and counted using a counting chamber. The cell suspension was again centrifuged for 5min at 210g and the pellet was resuspended in 800µl PBS per $2x10^6$ cells. The cells were transferred to a Gene Pulser® cuvette with 20µg linear vector.

The electroporation was performed by using a BIORAD Gene Pulser® II with 0.24kV and 0.500nF x 1000. The cell suspension was transferred to 5ml iPS-medium and distributed 1:5, 1:10, 2x1:20 and 2x1:30 to the 60mm dishes and incubated at 37°C, 5% CO_2, 95% rH.

Four factors

For the electroporation a MicroPorator was used because electroporation with the Gene Pulser was not possible. For each electroporation $1x10^5$ cells and 0.5µg linear vector were transferred into 10µl resuspension Buffer R.

Three different conditions were used:

Voltage	Pulse Width	Pulse Number
1000V	20ms	1
1400V	20ms	1
1700V	20ms	1

Table 2: Conditions for the four factors electroporation.

Then the electroporated cells were transferred to a 60mm dish containing 5ml iPS-medium.

5.4. G418 selection

The pEGFP-N3 vector carries a neomycin resistance cassette and therefore G418 can be used for selection. Two to three days after electroporation the iPS-medium was removed and iPS-medium G418 (4µl/1ml medium) was added. The selection medium was changed daily and the selection was performed for approximatively 10 days (depending on the state of the cells).

5.5. Picking of riPS-cells containing EGFP

Twelve days after electroporation the colonies were picked and expanded individually. The same protocol was used as picking up reprogrammed cell colonies.

The clones were expanded to 60mm dishes and at this point frozen and stored in liquid nitrogen.

6. Karyotype analysis

For the analysis 70% confluent T75 flasks were used. During 1h the cells were incubated in 10ml iPS-medium containing 100µl colcemid (0.01mg/ml) to arrest the cells at metaphase stage of mitosis. The following steps were achieved according to Triman *et al.* (1975).

7. Polymerase chain reaction

All the clones, four and three factors, were tested with PCR in order to analyse if the factors for reprogramming can be amplified. Confluent 35mm dishes were washed once with 2ml PBS and 500µl of 1x trypsin was added. The cells were incubated at 37°C for 5min. Then the cells were resuspended in 9ml SNL-medium and centrifuged for 4min at 210g. The pellet was resuspended in 2ml iPS-medium and transferred to 35mm dishes in order to let the SNL-feeders adhere. After 20min the solution containing more or less only riPS-cells was removed and transferred to a 15ml tube. The cells were centrifuged at 210g for 4min. The pellet was resuspended in 50µl lyses buffer (100mM Tris-HCl pH 8.00, 5mM EDTA, 100mM $MgCl_2$, 0.4% SDS) containing 1.25µl proteinase K (25µg/µl) and transferred to an Eppendorf tube. The tube was incubated 3h at 56°C and afterward 15min at 75°C in order to inactivate the proteinase K. After the incubation 50µl P/C/I (Phenol :Chloroform : Isoamylalcohol) was added to the samples, they were shortly vortexed and centrifuged for 5min at 16100g. The aqueous phase (on top) was collected and the DNA was precipitated with 2.5 Fällen buffer (50ml 100% EtOH containing 1.33ml 3M NaOAc pH 5.2) The solution was vortexed and incubated for at least 1h at -80°C.

The samples were centrifuged for 15min at 4°C, 16100g. The supernatant was removed and the DNA pellets were resuspended in 20µl water and used for the PCR.

All primers were designed with Clone Manager and ordered from Microsynth AG.

Primer	Sequence
pMX-S1811-forward	5'-GAC GGC ATC GCA GCT TGG ATA CAC-3'
mSox2-backward	5'-GCT TCA GCT CCG TCT CCA TC-3'
mOct4-backward	5'-TCT GAG CCT GGT CCG ATT CC-3'
mKlf4-backward	5'-CGC AGT GTC TTC TCC CTT CC-3'
m-c-Myc-backward	5'-GAG AAG AAG GAG CCT GAG CGA C -3'

Table 3: Sequences of all primers used for the PCR analysis of riPS cells.

1x Taq polymerase PCR reaction (20µl):

2µl Taq polymerase reaction buffer 10x

0.8µl 25mM $MgCl_2$

0.5µl 10mM dNTPs

1µl 10 µM forward primer

1µl 10 µM backward primer

0.1µl Taq Polymerase

4µl Q-solution 5x

8.6µl water

2 µl 1:10 diluted lysate

Positive control: 2µl reprogramming vector was used instead of 2µl lysate

Negative control: 2µl water was used instead of 2µl lysate

		Oct-3/4, Sox2	**Klf4**	**c-Myc**
Initial denaturation		95°C, 3min	95°C, 3min	95°C, 3min
Three-step cycle	Denaturation	95°C, 30s	95°C, 30s	95°C, 30s
	Annealing	62°C, 30s	58.7°C, 30s	60.2°C, 30s
	Elongation	72°C, 45s	72°C, 45s	72°C, 45s
	Number of three-step cycles	40	40	40
Final elongation		72°C, 5min	72°C, 5min	72°C, 5min

Table 4: PCR conditions.

In order to control the performance of PCR the probes were loaded on a 3% agarose gel.

20µl PCR product

4µl 6x loading dye

Out of this 10µl was loaded.

8. Testing marker of pluripotency

During cultivation the cells were characterized by testing the expression levels of typical pluripotency markers such as alkaline phosphatase, Oct-3/4 and SSEA-1.

8.1. Alkaline Phosphatase staining

This staining was done at several time-points of cultivation in iPS-medium.

The medium was removed and the cells were washed three times with 1x PBS. Afterwards the cells were fixed during 10min with 4% paraformaldehyde (PFA) and then washed three times 10min with 1x PBS.

After fixation the cells were washed twice for 10min using AP Buffer (100mM TrisCl pH 9.5, 100mM NaCl, 50mM $MgCl_2$). Then the cells were incubated for 1-2hs in AP-solution containing 0.5µl NBT (75mg/ml in 70% dimethylformamide) and 3.5µl BCIP (50mg/ml in 100% dimethylformamide) per 1ml AP-Buffer. After 1h, the AP expression was checked every 10min. Pluripotent cells appear blue, because they show a high expression of AP, whereas differentiated cells, such as feeders, remain uncoloured.

The staining was blocked with Tris-EDTA for 10min and the cells were then washed three times with PBT (1x PBS with 0.1% Triton) in order to eliminate precipitates.

Afterwards pictures of the colonies were taken.

8.2. Immunohistochemistry

The cells were fixed following the same protocol as for the AP staining.

The fixed cells were washed three times with PBT. Then the cells were incubated overnight at 4°C with the first antibodies diluted in PBT containing 4% horse serum. The day after the antibody-solution was removed and the cells were washed three times for 10min with PBT. The secondary fluorescence marked antibodies diluted in PBS were given to the cell either during a few hours on the shaker at 40rpm or overnight at 4°C. After the incubation the secondary antibodies were taken out and the colonies were washed twice for 10min with PBS.

	Primary antibody (dilution)	**Secondary antibody (dilution)**
three and four factors riPS-cells	Rabbit anti Oct-3/4 (1:500)	Alexa Fluor 594 goat anti rabbit IgG (1:500)
	Mouse anti SSEA-1 (1:100)	Alexa Fluor 488 goat anti mouse IgG (1:500)
three and four factors riPS-cells containing EGFP	Goat anti Oct-3/4 (1:250)	Cy5 donkey anti goat IgG (1:500)
	Mouse anti SSEA-1 (1:100)	Alexa Fluor 594 goat anti mouse IgG (1:500)

Table 5: Used antibodies for immunohistochemistry.

DAPI staining was also performed as a nuclei marker. Therefore the cells were incubated with DAPI (1:2000) attenuated in PBS at room temperature (RT). After 5min the cells were washed with PBS.

For taking pictures the fluorescent microscope was used.

9. Differentiation of riPS-cells *in vitro*

The differentiation potential was revealed by EB formation and induced differentiation to specific cell types *in vitro*.

9.1. Embryoid bodies formation

riPS cells were cultured in non-adhesive tissue culture plates in DMEM containing 10% FBS for one week.

9.2. Induced differentiation

Cells were plated on fibronectin-coated 35mm plates.

For neuronal induction the cells were cultured in DMEM/F12 (Invitrogen) with B27 supplement (Invitrogen). For myofibroblasts induction the cells were cultured in DMEM containing 10% FCS. After ten days of differentiation cells were fixed in 4% PFA and stained with βIII tubulin, glial fibrillary acidic protein (GFAP) and smooth muscle actin (SMA). Nuclei of the cells were stained with DAPI. Pictures were taken using the fluorescent microscope.

10. Teratoma formation

For teratoma formation $5x10^6$ riPS-cells were suspended in 1ml DMEM containing 10% FBS. 200µl ($1x10^6$ cells) suspension was then injected subcutaneously into each dorsal flank of NOD/SCID mice. Three weeks after the injection, teratomas were dissected and fixed in 4% PFA. Sections were prepared and stained with hematoxylin and eosin (HE-staining).

11. Blastocyst injections using three factors riPS cells

Day 1: The estrus of the donor females (Wistar) was assessed at day 1. All vaginal impedance measurements were made with the Estrus Cycle Monitor EC40 (Fine Science Tools, Foster City, CA.), as previously described (Ramos et al., 2001). The technique is based on the fact that the electrical impedance of the epithelial cell layer of vaginal mucosa is significantly higher during the estrus than in the other stages of the estrus cycle. A value equal or higher 8kΩ is considered positive.

Rats in estrus were mated with fertile male Brown Norway male rats.

Day 2: The donor females and the male rats were separated.

The estrus for the foster mothers (Wistar) was assessed at day 2, using the Estrus Cycle Monitor EC40. The positive females were mated with vasectomised male Wistar male rats.

Day 3: The foster mothers and the vasectomised male rats were separated.

Day 4: At day 4 a vial of electroporated three factor riPS cells was thawed and plated in one 60mm plate using 5ml of iPS-medium.

Day 6: At E4.5 blastocysts were flushed from the uterus with M2-medium and cultured for 2hs in KSOM-medium. During the blastocyst cultivation the riPS cells were trypsinised and transferred to a 100mm dish in order to let the SNL-feeders adhere. The riPS cells were then transferred into drops of HEPES-buffered iPS-medium (Injection-medium). After cultivation in KSOM-medium the blastocysts were transferred into drops of M16-medium. About 10-15 riPS cells were injected into each blastocyst. After a recovery period of about 2hs, injected blastocysts were transferred into the uterine horns of the pseudo pregnant Wistar foster mothers.

	Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**
Donors	♀ Wistar In estrus X ♂ Brown Norway	E0.5 Separation	E1.5	E2.5	E3.5	E4.5 Blastocyst isolation Cultivation Blastocyst Transfer
Foster		♀ Wistar In estrus X Vasectomised ♂ Wistar	E0.5 Separation	E1.5	E2.5	E3.5 Blastocyst Transfer
Cel				riPS cells → 6cm		

Table 6: Timetable for mating the donors and foster mothers and blastocyst transfer.

D. Results

1. Reprogramming

1.1. Maxi Preparation

Four DH5α bacterial strains, each containing a vector encoding one of the 4 murine factors for Oct-4, Sox2, Klf4 and c-Myc, were used for a maxi DNA preparation. The vectors were amplified and isolated and the DNA concentration was measured by spectrophotometry (Table. 1).

Maxi preparation	DNA concentration (µg/µl)
mOct-4	1.095µg/µl
mSox2	0.89µg/µl
mKlf4	1.235µg/µl
m-c-Myc	0.915µg/µl

Table 1: DNA concentration of maxi preparation of the reprogramming vectors.

1.2. Reprogramming of rat embryonic fibroblasts and picking of riPS cell colonies

The retroviruses containing the four reprogramming factors were produced by transfecting Plat-E cells with the appropriate vector. PlatE cells contain all the elements necessary for viral production and packaging and they start viral production when transfected with the vectors. The retroviruses containing the vectors encoding for the reprogramming factors were released in the medium after the death of the PlatE cells. The virus-containing medium was then used to infect rat embryonic fibroblasts (REFs) (day 0). REFs were either infected with four factors, Oct-4, Sox2, Klf4 or c-Myc (riPS-4) or with three factors, Oct-4, Sox2 and Klf4 (riPS-3).

After 24hs of infection the medium was replaced with fresh FP-medium, from day 2 after the infection the cells were cultivated with iPS medium instead of FP-medium.

The resulting riPS cells were cultivated until the colonies became big enough to be picked (Table 2). At this point the colonies showed an ES-cell like morphology (Fig. 1A + B).

riPS-cells	Days of picking
riPS-4 cells	Day 17, Day 18
riPS-3 cells	Day 20, Day 21

Table 2: Day of picking after infection of REFs.

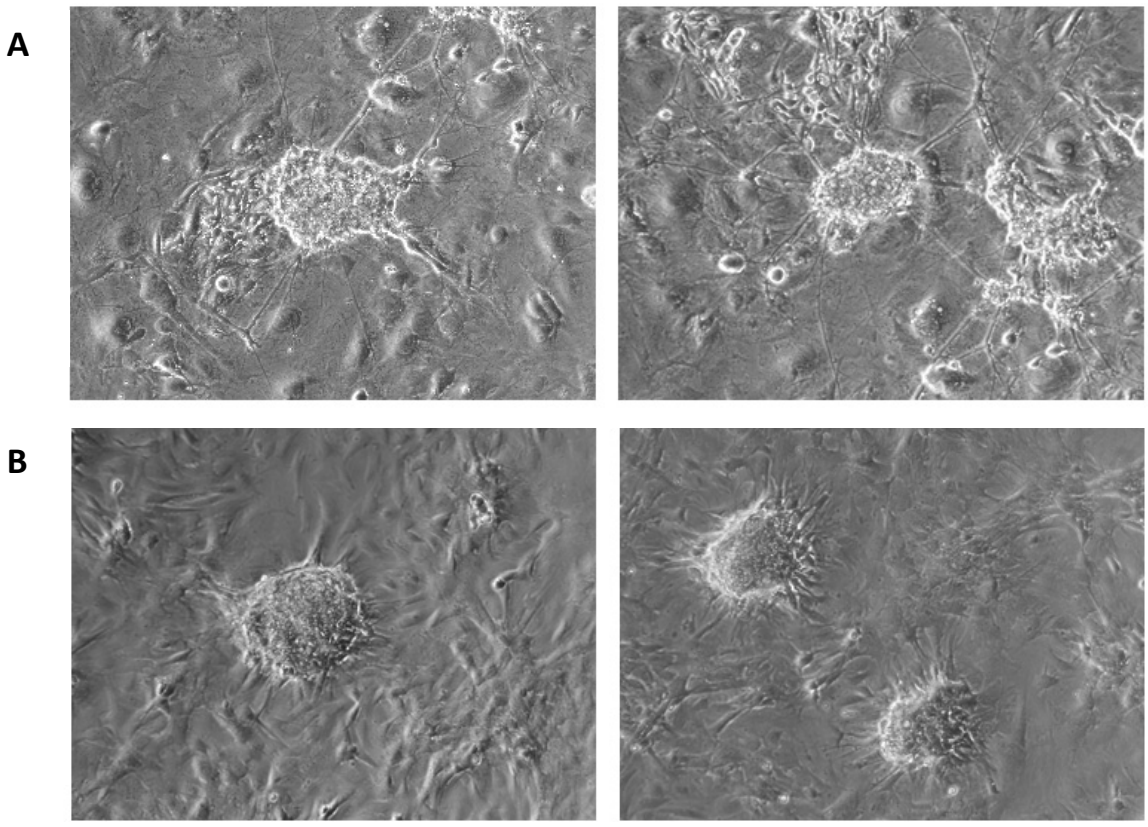

Fig. 1: Morphology riPS-4 (**A**) and riPS-3 (**B**) colonies at the day of picking. The cells grow in multilayered round colonies with a shiny halo, and have a morphology which is very similar to ES cells. Magnification: 10x

2. Cultivation of riPS cells

After picking the riPS cells were expanded on SNL feeders. During expansion the riPS-4 cells and riPS-3 cells were also electroporated with a vector encoding EGFP (pEGFP-N3). Before and after electroporation the riPS cells several characterisation analyses were performed in order to examine their pluripotency (Table 3). The following analyses were performed

- Karyotype analyses
- PCR
- Alkaline phosphatase staining
- Immunohistochemistry (IHC) for Oct-3/4 and SSEA-1
- Analysis of the differentiation potential
    - *In vitro*: EB formation and differentiation into different cell types
    - *In vivo*: Teratoma formation
- Blastocyst injection

These assessments were started after passage 8, and were repeated at different time points during expansion.

	Before electroporation		After electroporation **	
Analyses	**riPS-4**	**riPS-3**	**riPS-4**	**riPS-3**
Karyotype	X	X	-	X
PCR	X	X	X	X
AP staining	* X	X	X	X
IHC	* X	X	X	X
EB formation	X	X	-	-
Differentiation *in vitro*	X	X	-	-
Teratoma formation	X	X	-	-
Blastocyst injection	-	-	-	*** X

Table 3: Summary of the performed analyses of the different riPS-cells.

* AP staining and IHC were performed for three clones: 4B1, 4B5 and 4C1. The clone 4B5 was then expanded and used for further analyses and electroporation.

The riPS-3 clone 3F6 was used for all the analyses and also for electroporation.

** For the analyses after electroporation the riPS-4 clones 4A3, 4A6, 4B3 and 4B5* and the riPS-3 clones 3A1, 3A2 and 3A6 were used.

*** The blastocyst injection was performed with the clone 3A1.

- not performed

2.1. Morphology of riPS cell colonies

The morphology of the cells in culture allows an assumption about their pluripotent state.

During cultivation riPS cells showed a morphology similar to the one of ES cells, characterized by a round shape, shiny borders and a growth in compact, multilayer colonies (Fig. 2).

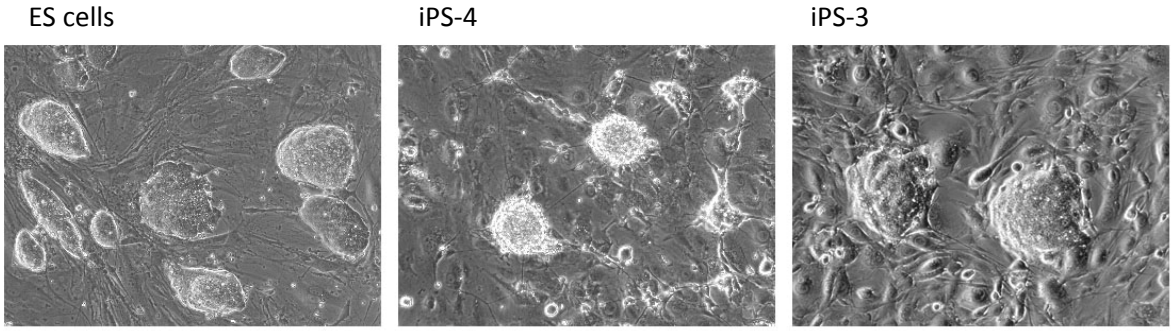

Fig. 2: Morphology of ES cells, riPS-4 and riPS-3 cells. Magnification:

2.1.1. riPS-4 cells in culture

riPS-4 cells change their morphology during cultivation, but they keep their pluripotent character in each stadium (Fig. 3).

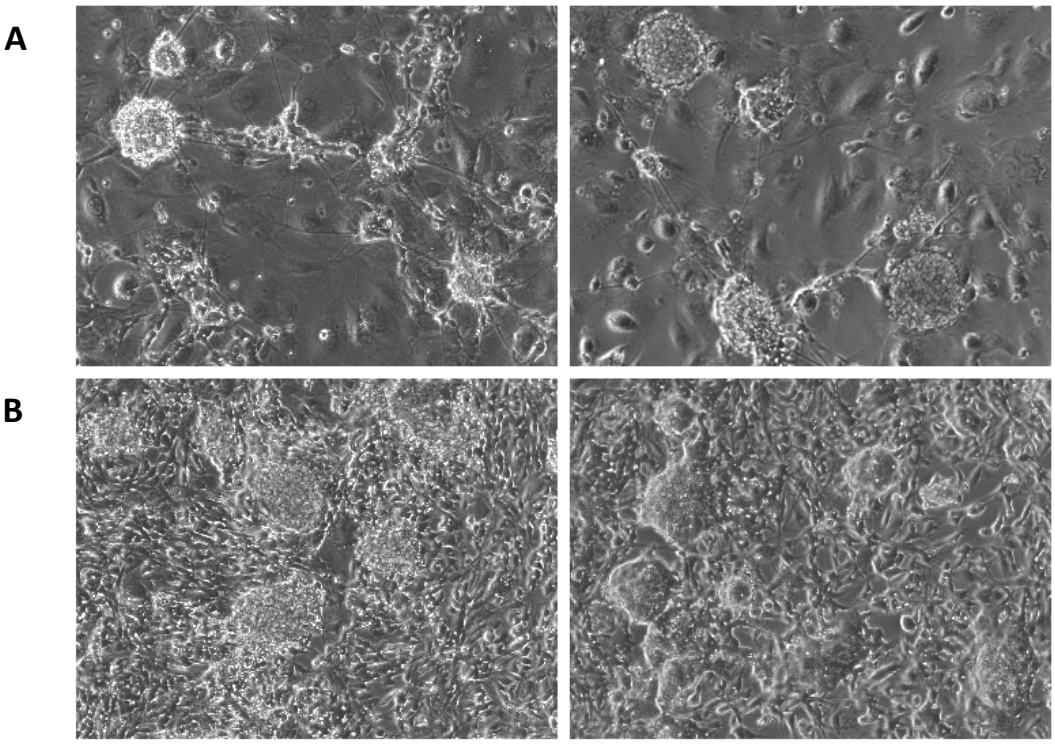

Fig. 3: Morphology of riPS-4 cells at different passages. **A)** Early passages. **B)** Later passages.
The colonies maintain an ES cell-like morphology. Magnification: 10x

Furthermore, riPS-4 cells needed about four days in culture after splitting to form again ES cell-like colonies (Fig. 4).

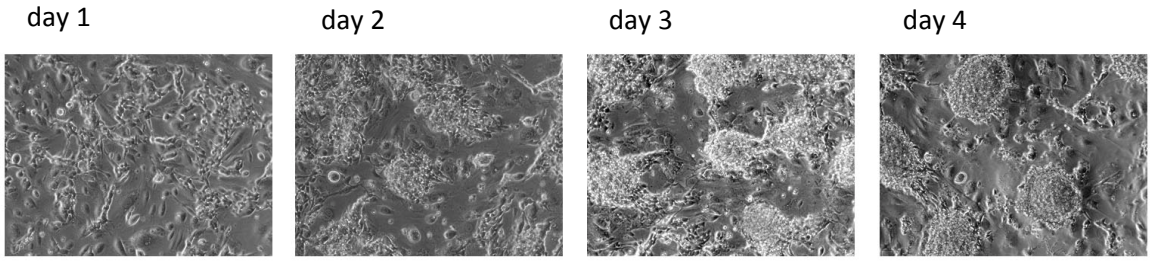

Fig. 4: Behaviour of riPS-4 cells after splitting. Magnification: 10x

2.1.2. riPS-3 cells in culture

At early passages, until passage 8, riPS-3 cells formed small and compact colonies. From passage 8 on they exhibited a morphology similar to ES-cells (Fig. 5). Analyses such as PCR, AP staining and immunohistochemistry were performed after passage 8.

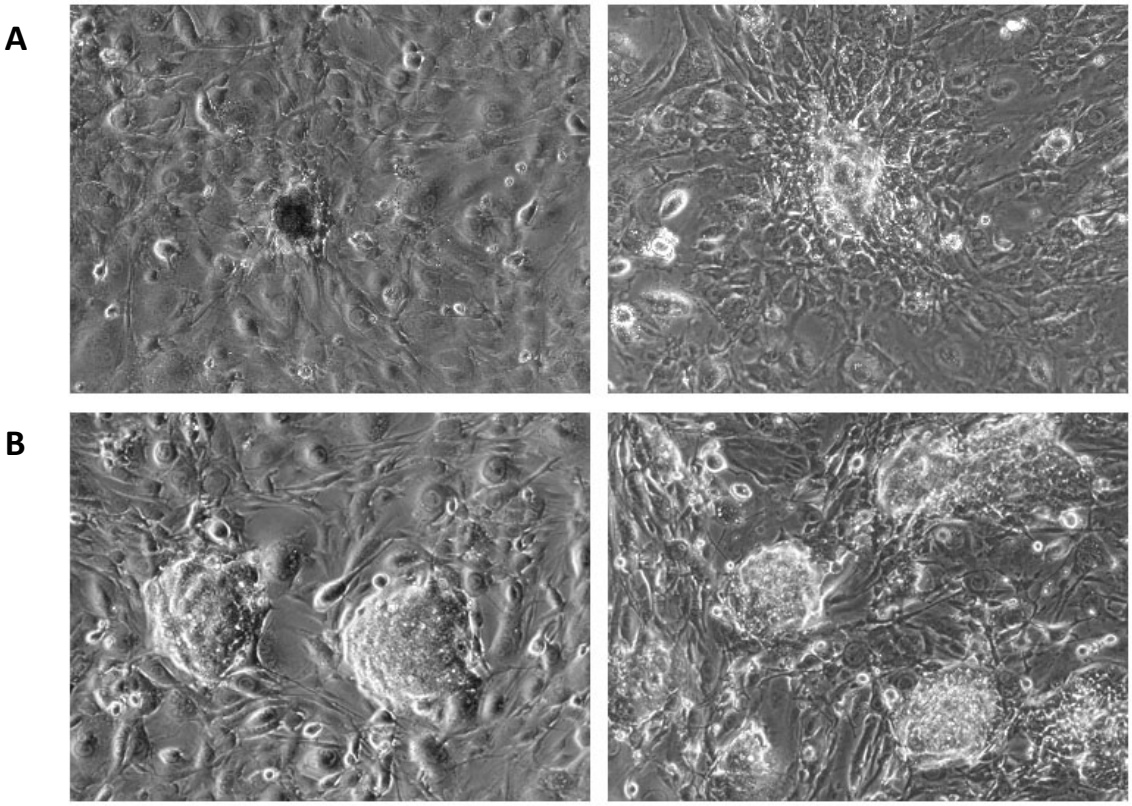

Fig. 5: **A)** The morphology of riPS-3 colonies at early passages. **B)** riPS-3 colonies after passage 8.
These colonies show an ES cell-like morphology, with shiny borders and a round shape. Magnification: 10x

2.2. Electroporation of riPS-4 cells and riPS-3 cells

Electroporation is a mechanical method used to introduce polar molecules into a host cell through the cell membrane. In this procedure, an electric pulse temporarily disturbs the phospholipid bilayer, allowing the DNA molecules to enter in the cell.

For this purpose the vector must be linearised before being electroporated into the riPS cells, allowing a random integration into the genomic DNA.

The pEGFP-N3 vector was cut with the restriction enzyme SacI, which cuts in the multiple cloning site (MCS).

During expansion, riPS cells were electroporated with the linearised vector, when confluence in a 60mm dish was achieved. For the electroporation the riPS-4 clone 4B5 and the riPS-3 clone 3F6 were used.

Two to three days after electroporation the cells were selected for about 10 days with iPS medium containing G418. During the selection cells that did not contain the vector died because they lacked the neomycin resistance.

Twelve days after electroporation, the green fluorescent colonies (Fig. 6) were picked and expanded.

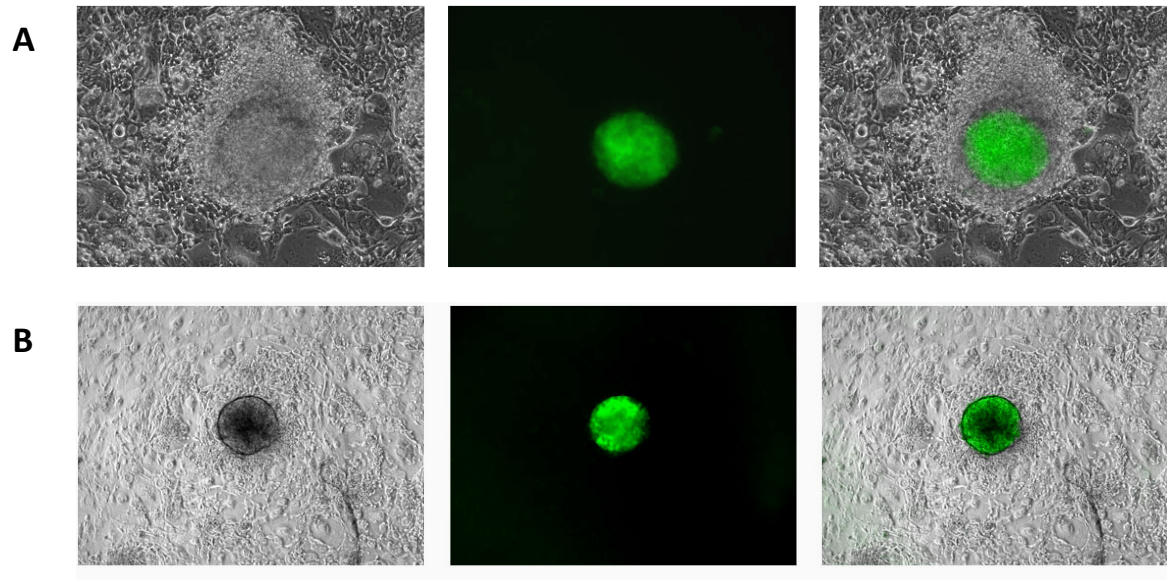

Fig. 6: An example of a riPS-4 colony **A)** and a riPS-3 colony **B)** which were picked after twelve days of G418-selection and then expanded. Magnification: 10x

2.3. Karyotype analyses

A karyotype analysis was performed for both the riPS-4 and riPS-3 cells before and after electroporation. Rat cells contain 42 chromosomes, some of which are telocentric and some are metacentric (Fig. 7). All the analysed clones contained an average of 42 chromosomes, after 40 rounds of counting.

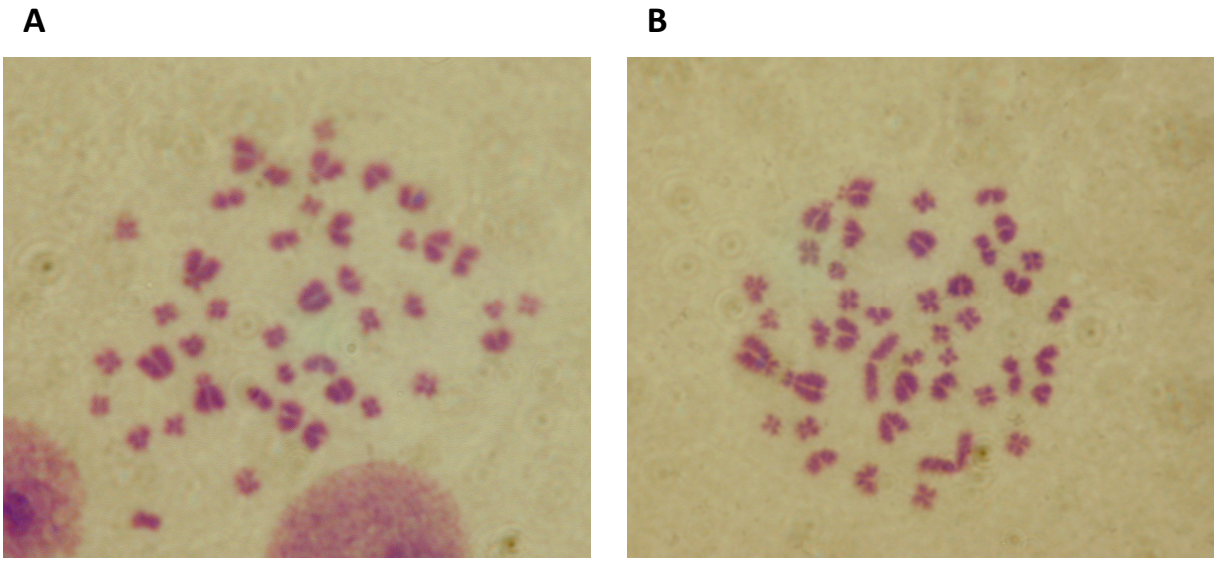

Fig. 7: Chromosome spreads of a cell from the 4B5 clone (**A**) and of the 3F6 clone (**B**). Magnification: 100x

2.4. PCR analyses before and after electroporation

riPS clones were screened before and after electroporation for the presence of the introduced reprogramming factors, Oct-4, Sox2, Klf4 and c-Myc. The PCRs were performed with a forward primer which binds on the promoter pMX-S1811 of the pMXs-GW vector and a backward primer which binds on the specific sequence of the reprogramming factor.

The products were visualized by loading the PCR reactions on a 3% agarose gel (Fig. 8 + Fig. 9).

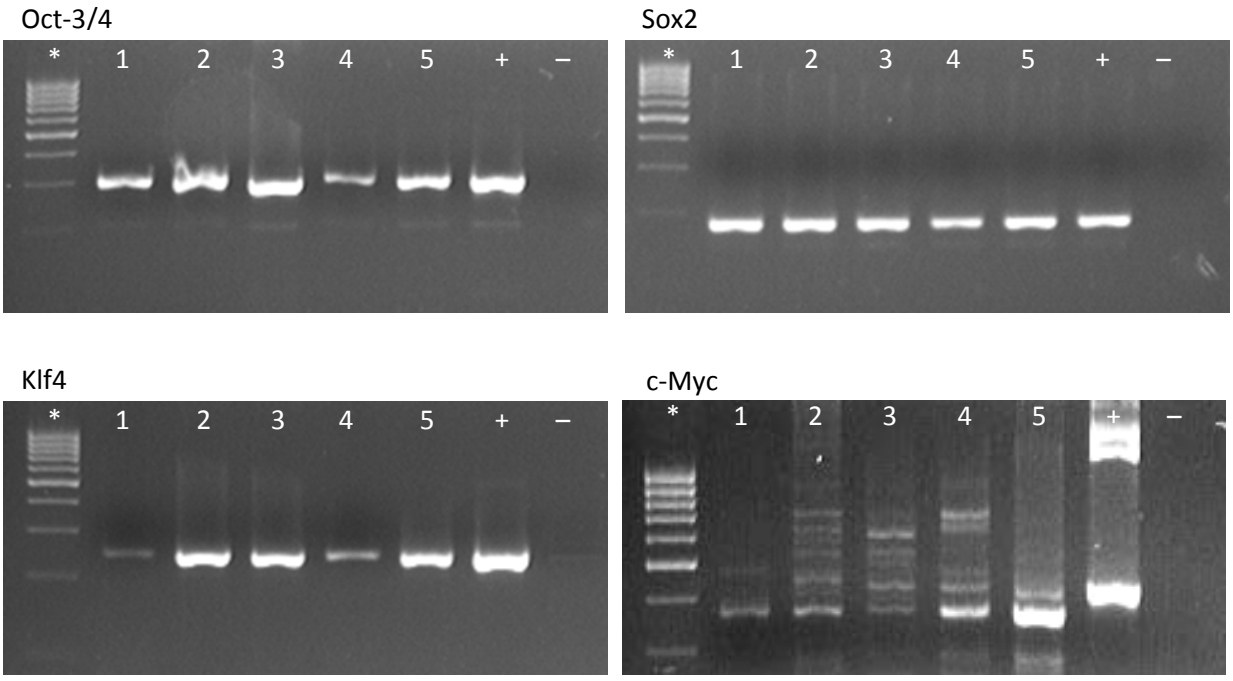

Fig. 8: PCR analysis of riPS-4 cells for the reprogramming factors. (**5**) 4B5 represents the riPS-4 clone before electroporation with the EGFP vector and (**1**) 4A3, (**2**) 4A6, (**3**) 4B3 and (**4**) 4B5* represents the expanded clones, from the electroporated 4B5 clone, after electroporation. The pMXs-vectors encoding the four transgenes were amplified and used as a positive (**+**) control and water was used as a negative control (**–**).The PCR analysis showed, that the riPS-4 clones before and after electroporation contained all the four reprogramming factors.

(*****) DNA ladder low range

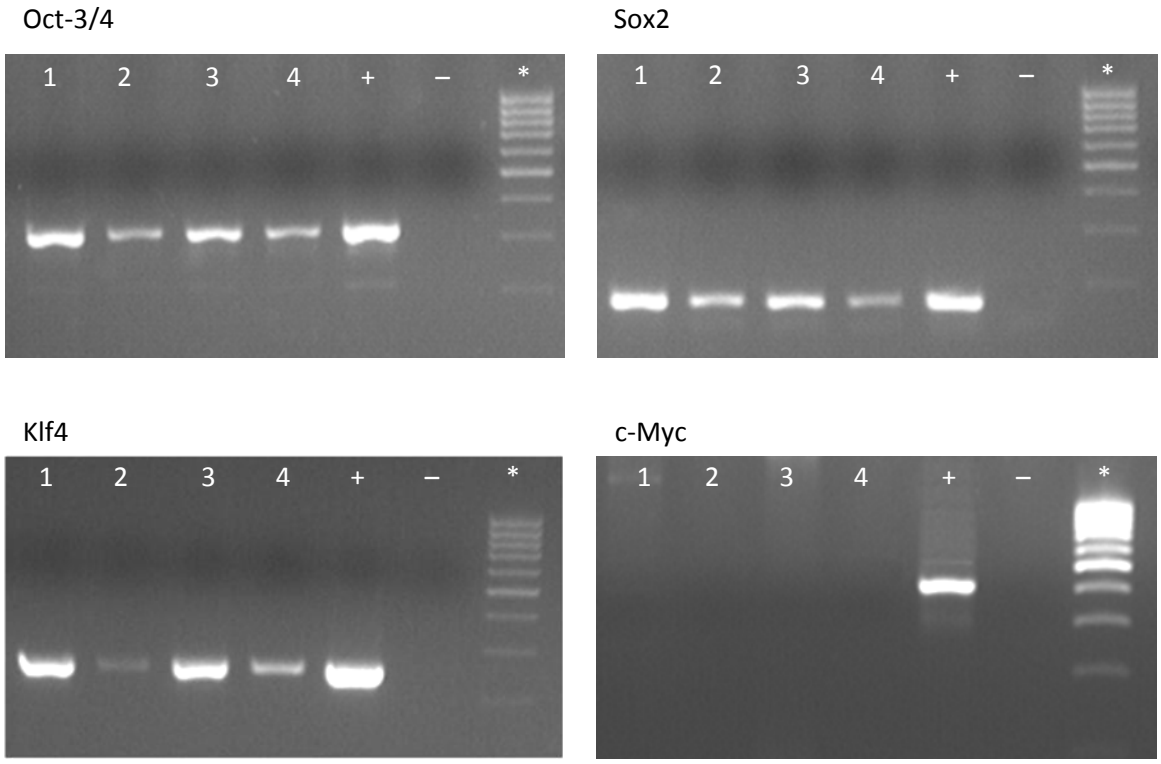

Fig. 9: PCR analysis of riPS-3 cells for the reprogramming factors. (**1**) 3F6 represents the riPS-3 clone before electroporation with the EGFP vector and (**2**) 3A1, (**3**) 3A2 and (**4**) 3A6 represents the expanded clones from the electroporated 3F6 clone after electroporation. The pMXs-vectors encoding the four transgenes were amplified and used as a positive (**+**) control and water was used as a negative control (**–**). The PCR analysis showed that the riPS-3 clone before and after electroporation contain the three introduced factors Oct-3/4, Sox2 and Klf4.
(*****) DNA ladder low range.

2.5. Alkaline phosphatase staining and immunohistochemical analyses

2.5.1. Alkaline phosphatase staining

Pluripotent stem cells have a high expression of alkaline phosphatase; they therefore become blue after a treatment with AP-buffer containing NBT and BCIP. Differentiated cells and SNL feeders were not stained and therefore appeared uncolored.

The riPS-4 clones and riPS-3 clones before and after electroporation were tested for AP activity at different time points during cultivation (Fig. 10 + Fig 11).

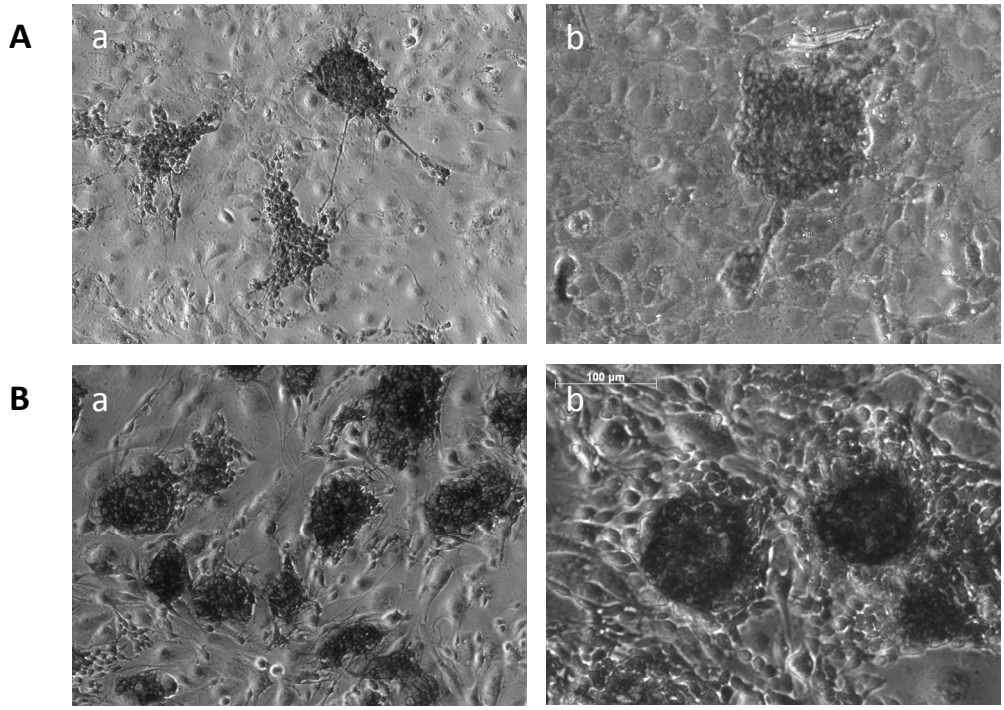

Fig. 10: **A)** riPS-4 colonies, clone 4B5, before electroporation. **B)** riPS-4 colonies, clone 4B3, after electroporation. The riPS-4 clones maintained the typical characteristics of pluripotent cells and were stained not affected by electroporation. Magnification: (**a**) 10x / (**b**) 20x

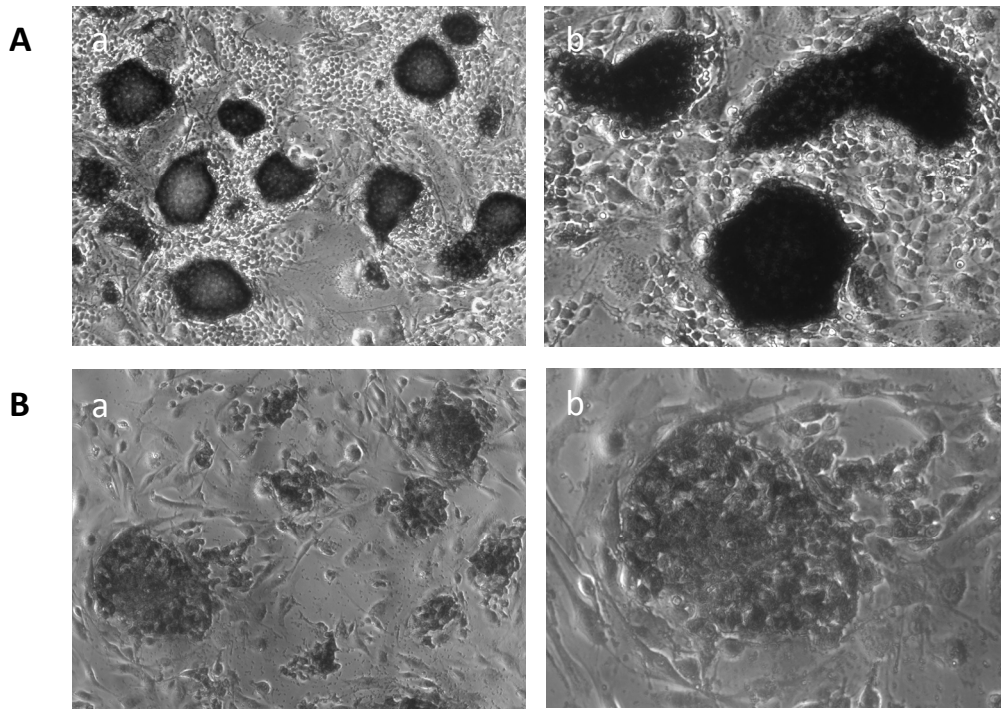

Fig. 11: **A)** riPS-3 colonies, clone 3F6, before electroporation. **B)** riPS-3 colonies, clone 3A1, after electroporation.. The riPS-3 clones maintained the typical characteristics of pluripotent cells and were stained not affected by electroporation. Magnification: (**a**) 10x / (**b**) 20x

2.5.2. Immunohistochemical analyses: Oct-3/4 and SSEA-1

Oct-3/4 and SSEA-1 are typical markers of pluripotency as described in the introduction. By using anti Oct-3/4 and anti-SSEA-1 antibodies and secondary antibodies coupled with a dye it is possible to check whether the cells express these markers.

Oct-3/4 is only expressed in the nuclei of undifferentiated cells and SSEA-1 is expressed on the surface of undifferentiated cells. A DAPI staining was also performed as a control in the riPS cells before electroporation. Given that the electroporated cells are green due to the EGFP expression and that we stained Oct-3/4 in blue and SSEA-1 in red, we could not use another color for the DAPI staining. We therefore did not perform any DAPI staining. DAPI forms fluorescent complexes with dsDNA, rendering thereby the nuclei of both feeders and riPS cells visible.

In Fig.12, Fig. 13, Fig. 14 and Fig. 15 the resulted IHC of the riPS cells are shown.

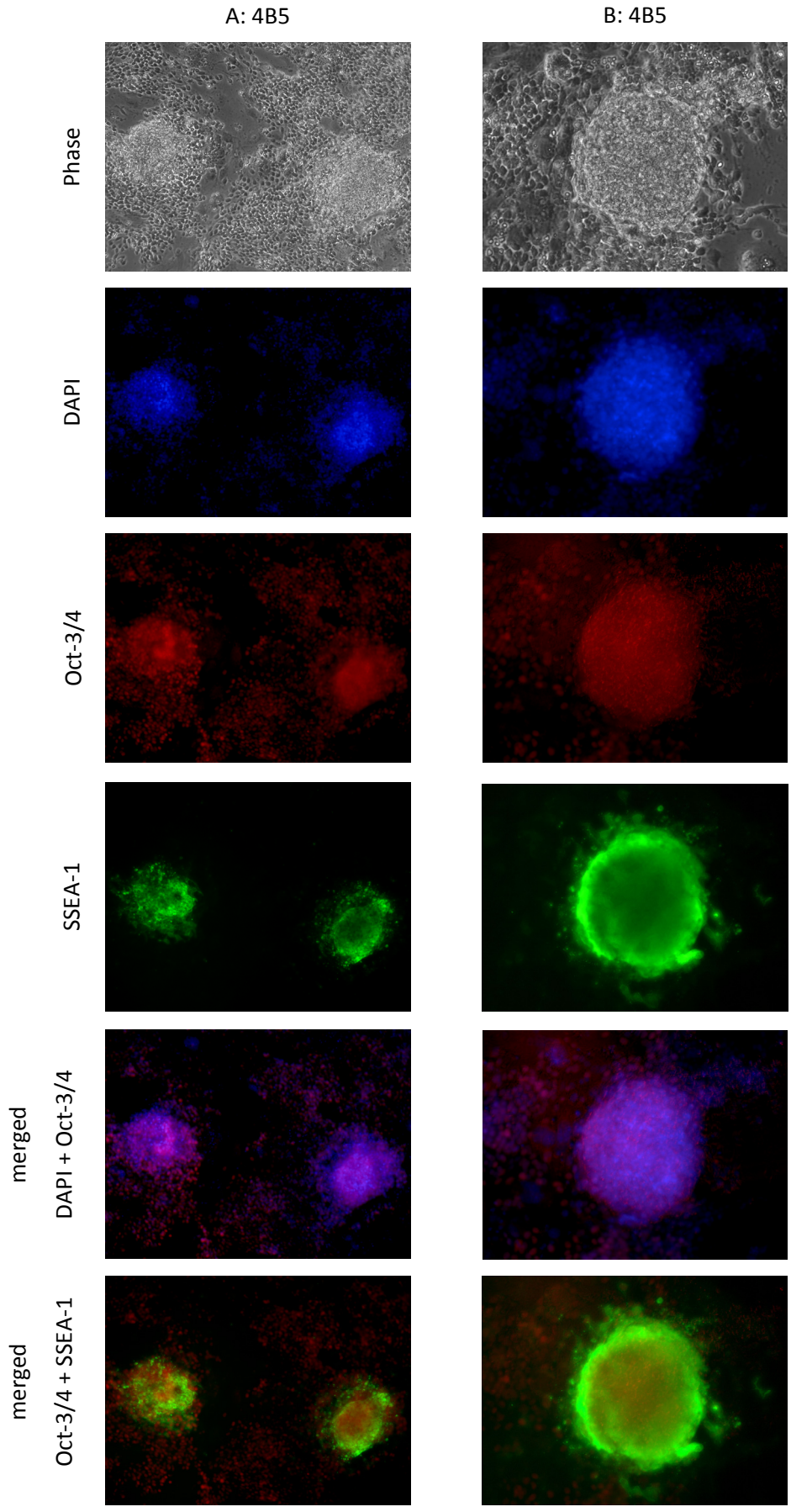

Fig. 12: riPS-4 clone 4B5 before electroporation. Magnification: (**A**) 10x (**B**) 20x

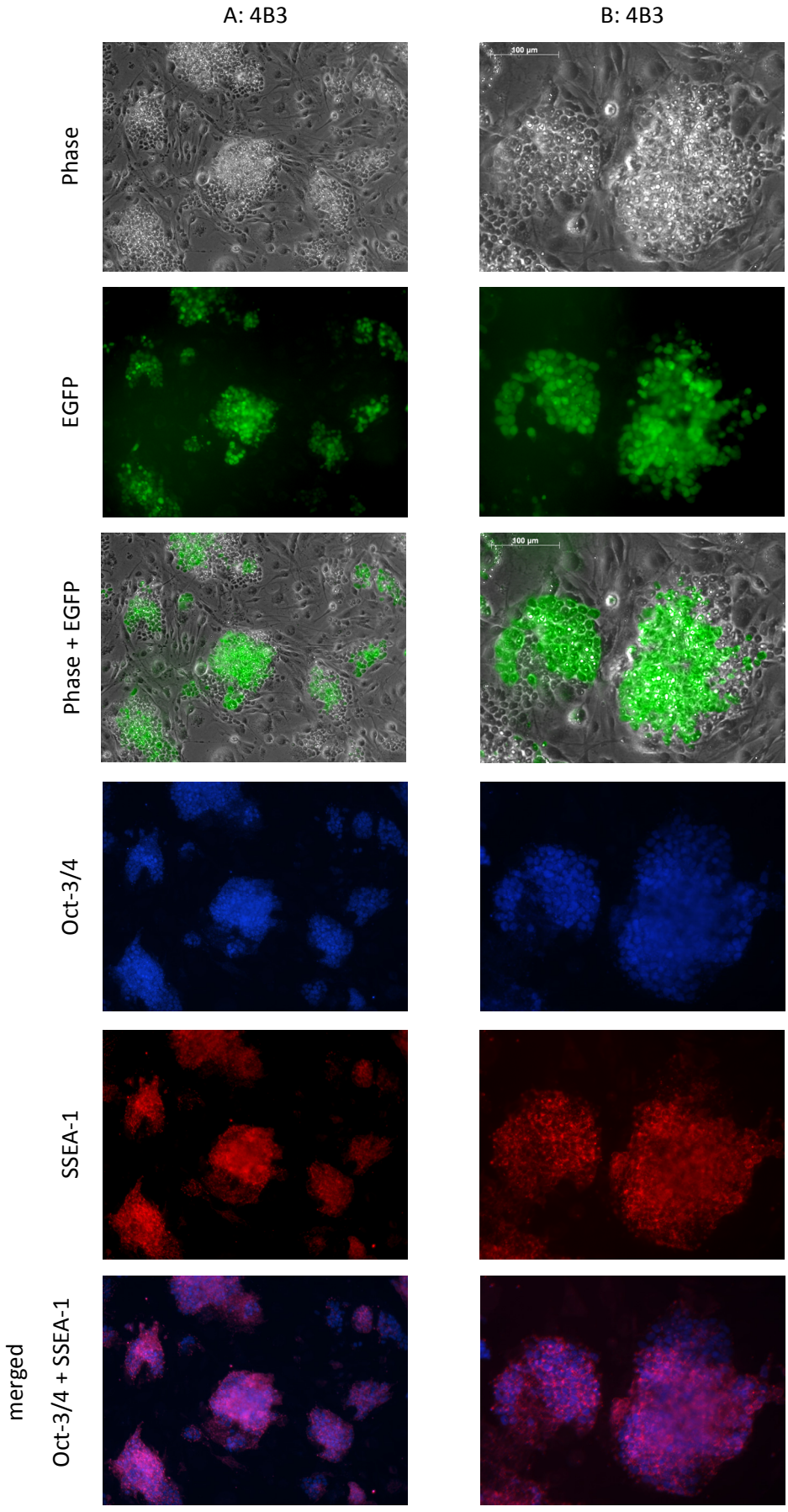

Fig. 13: riPS-4 clone 4B3 after electroporation. Magnification: (**A**) 10x (**B**) 20x

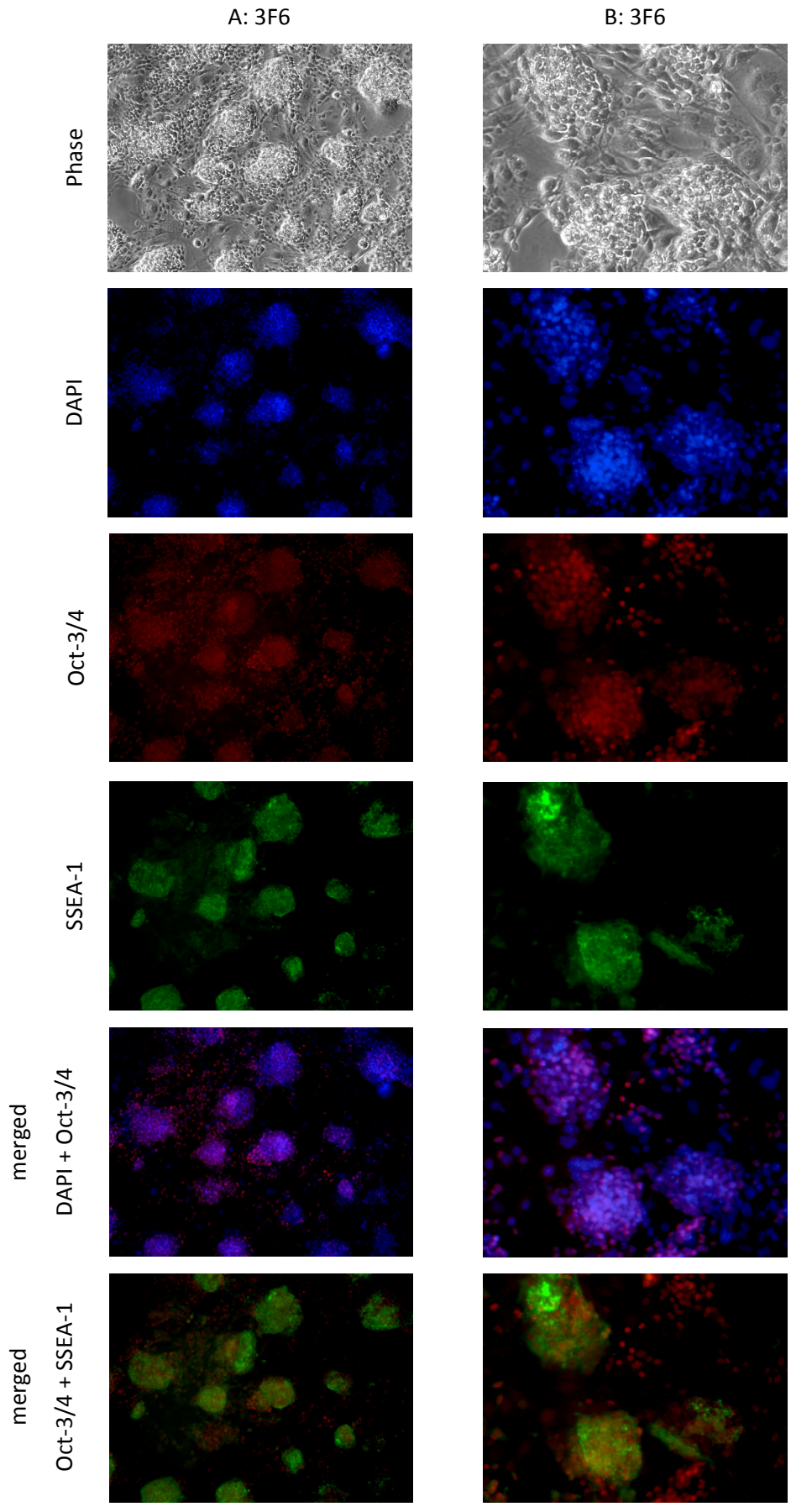

Fig. 14: riPS-3 clone 3F6 before electroporation. Magnification: (**A**) 10x (**B**) 20x

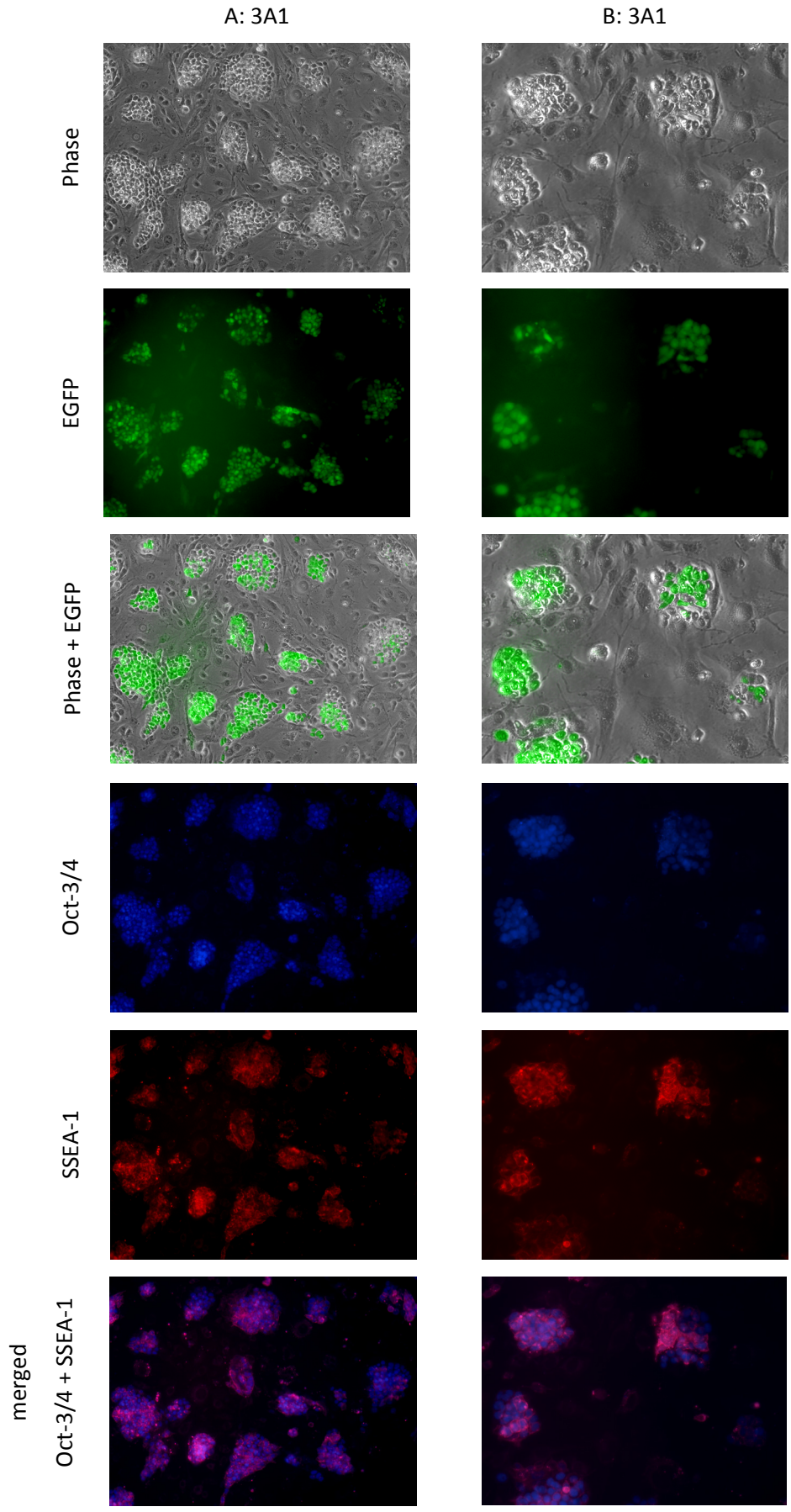

Fig. 15: riPS-3 clone 3A1 after electroporation. Magnification: (**A**) 10x (**B**) 20x

2.6. Differentiation potential *in vitro*

2.6.1. Embryoid bodies formation

riPS-4 and riPS-3 cells were cultured in non-adhesive tissue culture plates containing DMEM + 10% FBS. Both, the riPS-4 and riPS-3 cells, formed EB (Fig. 16).

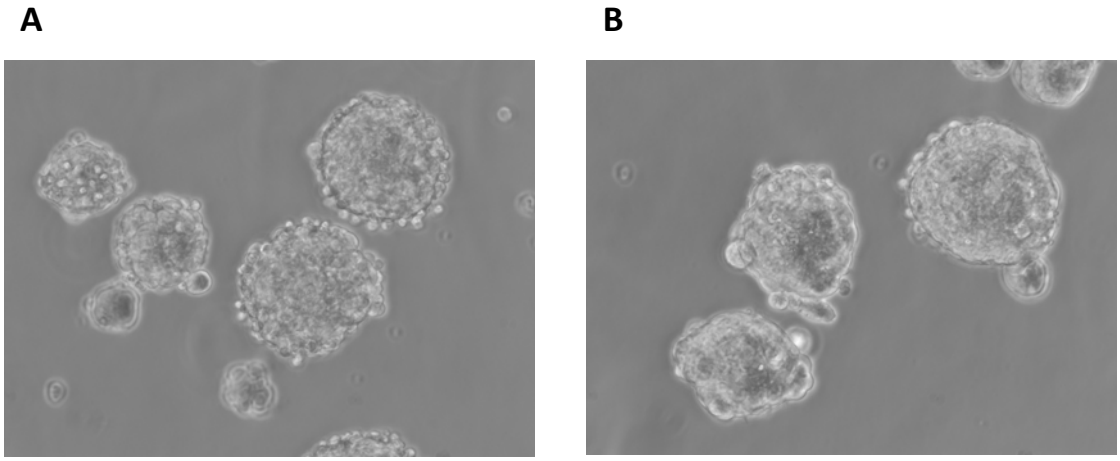

Fig. 16: **A)** EB formed by riPS-4 cells. **B)** EB formed by riPS-3 cells. Magnification: 20x

2.6.2. Differentiation *in vitro*

riPS-4 and riPS-3 cells were trypsinized and plated on fibronectin-coated plates. To initiate differentiation to neuronal cell types, the riPS-4 and riPS-3 cells were cultured in DMEM/F12 containing B27 supplement. To differentiate the cells to myofibroblasts, they were cultivated in DMEM containing 10% FCS.

After ten days of culture, immunostainings were performed, to detect cells, which are positive for βIII tubulin (ectoderm marker), glial fibrillary acidic protein (ectoderm marker, GFAP) or smooth muscle actin (mesoderm marker, SMA) (Fig. 17).

During differentiation to neuronal cell types we observed that the riPS cells first formed ganglia and then build neurites.

Differentiation to derivatives from the endoderm was not performed due to the absence of a protocol to induce differentiation to endodermal cell types *in vitro*.

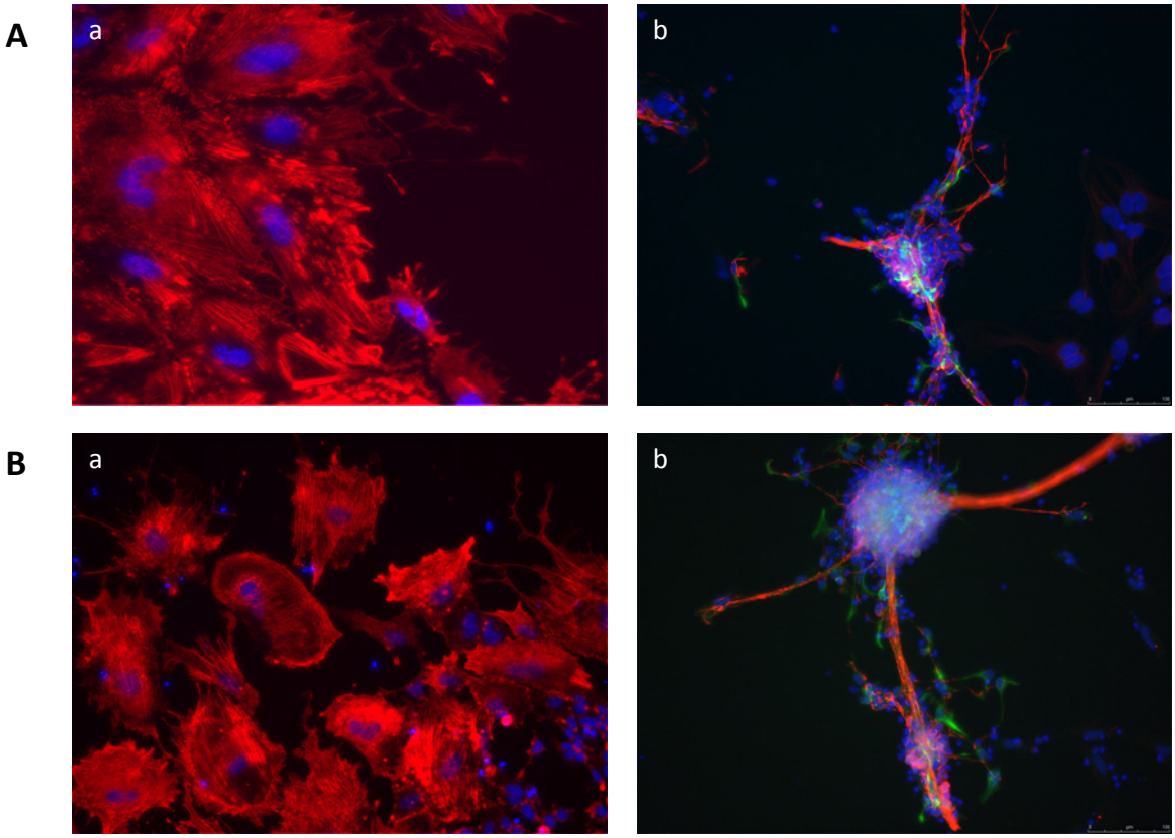

Fig. 17: Differentiation of riPS-4 (**A**) and riPS-3 (**B**) cells *in vitro*. Immunofluorescence staining confirmed differentiation into smooth muscles (red = SMA, blue = DAPI) (**a**), neurons and glial cells (red = βIII-tubulin, green = GFAP, blue = DAPI) (**b**). Magnification: 20x

2.7. Differentiation potential *in vivo*

2.7.1. Teratoma formation

riPS cells were subcutaneously injected into nude and NOD/SCID mice. Three weeks after injection of the riPS-4 and riPS-3 cells, tumor formation was observed for both cell types (Fig. 18 A + B).

Histological examination of the resulted riPS-3 cell teratoma revealed that the cells differentiated into various tissues of the three germ layers (Fig. 18 C + D + E).

Histological examination of the tumors induced by the injection of riPS-4 cells has not yet been performed.

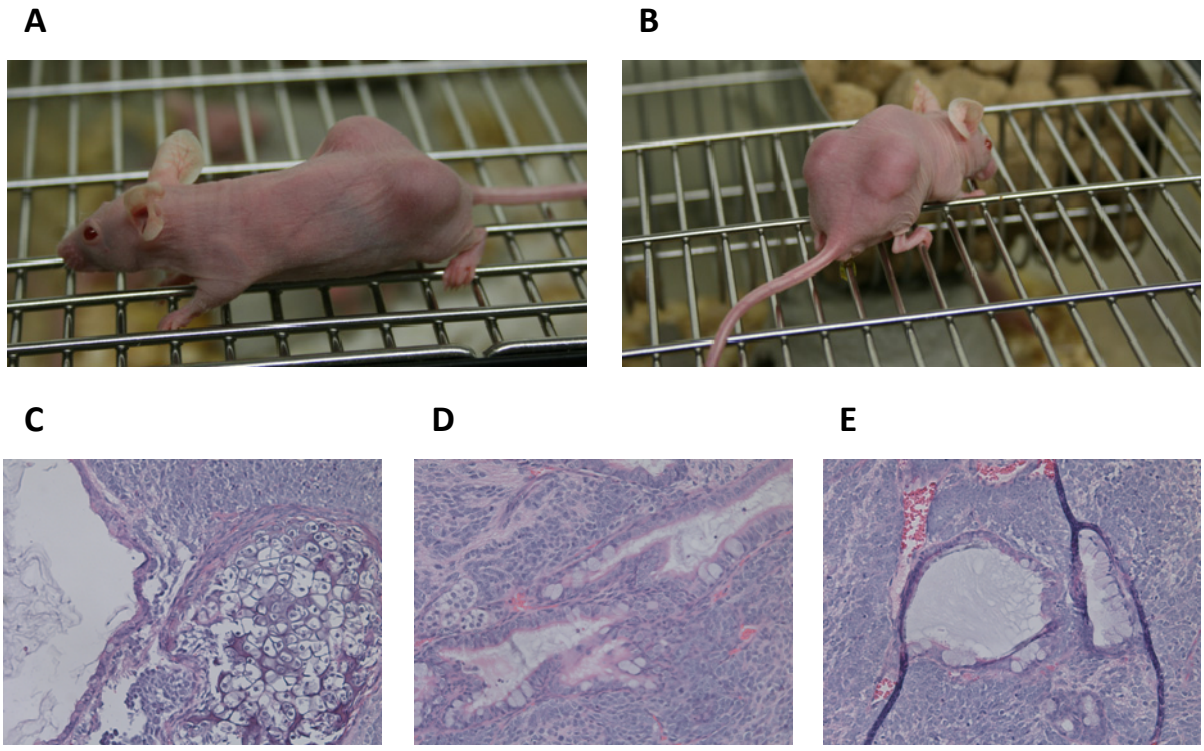

Fig. 18: (**A–B**) Teratoma formation in nude mice after injection of riPS cells. (**C–E**) Histological sections with HE-staining of teratomas derived from riPS-3 cells. The teratomas contained tissues derived from all three germ layers including cartilage (mesoderm) (**C**), epithelium (ectoderm) (**D**) and glands (endoderm) (**E**).

2.7.2. Blastocyst injection

Green fluorescent riPS-3 cells from the electroporated clone 3A1 (Fig. 19) were injected into blastocysts (Fig. 20) and transferred into foster mothers. Out of 3 injection rounds, 6 foster mothers were implanted, but only one litter was obtained. Because the EGFP was difficult to detect, for unclear reasons, a PCR analysis with hair from the newborns was performed in order to detect the presence of EGFP positive hair follicles (Fig. 21).

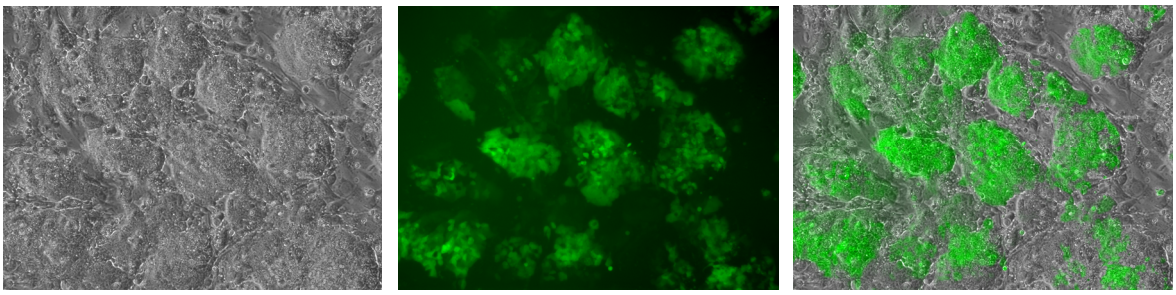

Fig. 19: Electroporated clone 3A1. This clone was used for blastocyst injection.

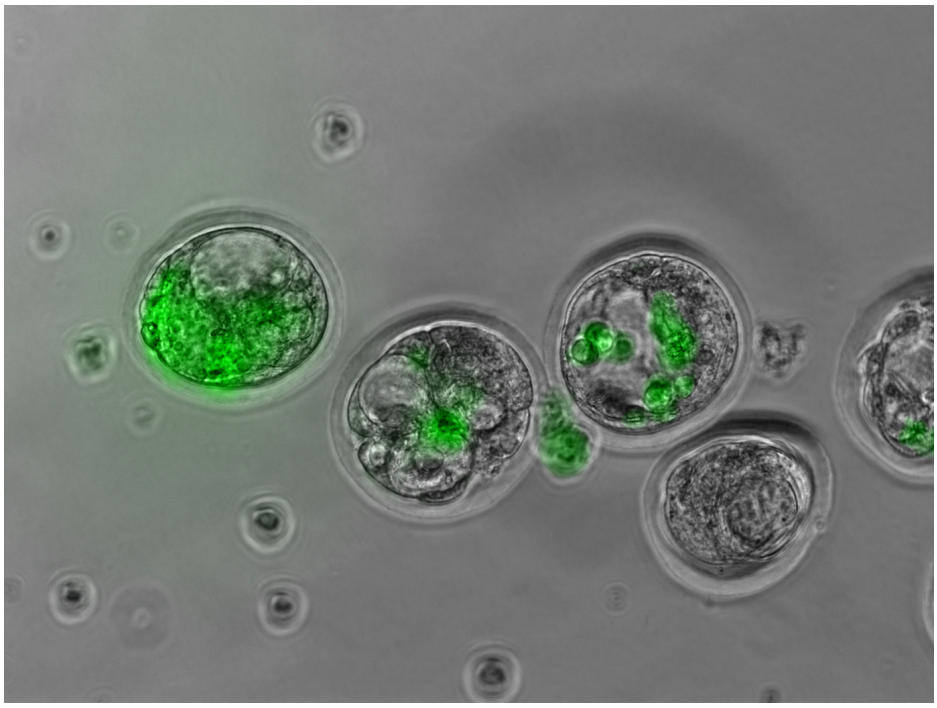

Fig. 20: Blastocysts after injection of green fluorescent 3A1 cells.

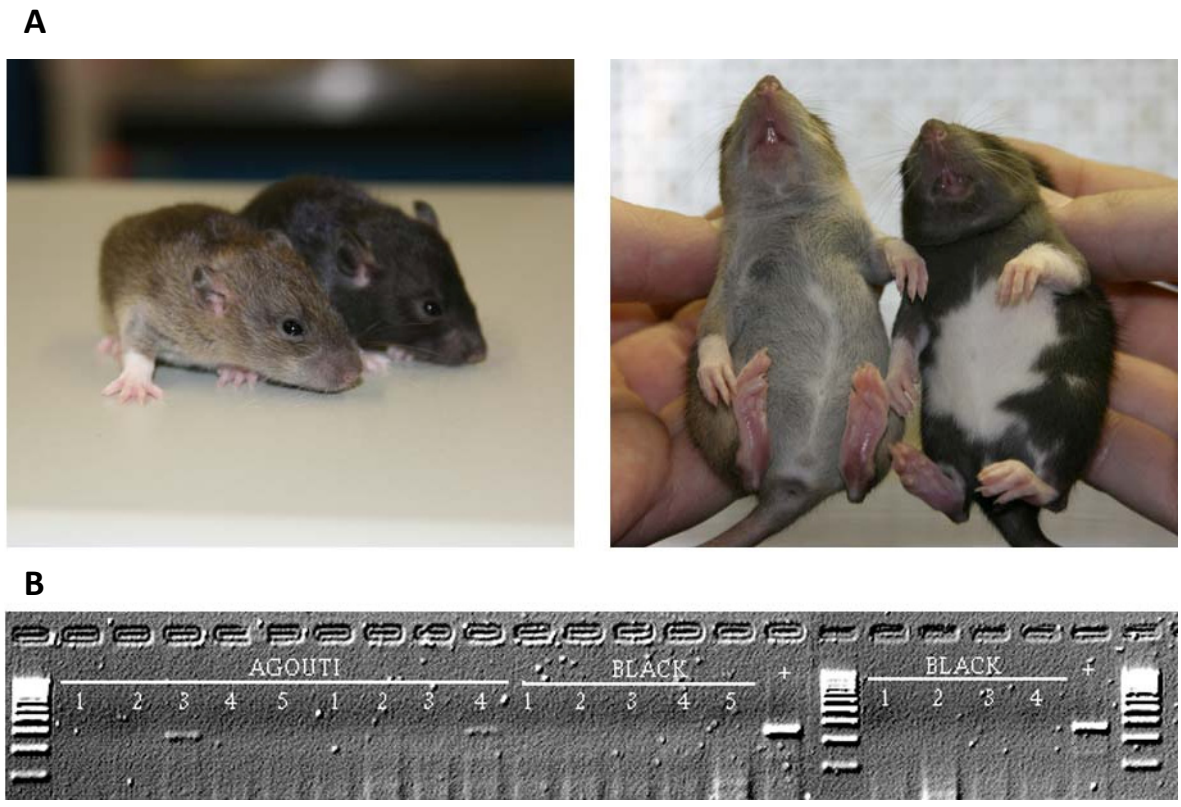

Fig. 21: **A)** Chimeric rats obtained after injection of riPS-3 cells clone 3A1. **B)** PCR for EGFP. The agouti rats were positive were as the black ones were negative. (**1-5**) Hair from different body parts from one rat. (**+**) Positive control = genomic DNA from clone 3A1 cells.

3. Reprogramming of knock-out fibroblasts

As described in the introduction it is very difficult to generate a KO rat. To date the generation KO rats by HR in pluripotent stem cells still has not been successful.

In our laboratory Oliver Sterthaus (see section 4.2.2. / Introduction) succeed in generating KO fibroblasts for the Neurotrypsin gene.

After the successful reprogramming of embryonic rat fibroblasts and the subsequent confirmation of their pluripotent state proven by the experiments described above, we decided to reprogram the rat KO fibroblasts. The possible resulting rat KO iPS cells will be used for the generation of KO rats.

Our experiments also showed, that the REFs can be reprogrammed to a pluripotent state by Oct-3/4, Sox2 and Klf4 in the absence of the oncogene c-Myc. We therefore decided to reprogram the KO fibroblasts only with the three factors Oct-3/4, Sox2 and Klf4.

24 days after infection, KO riPS-3 colonies with an ES cell-like morphology (Fig. 22) were picked. After infecting 250'000 plated KO fibroblasts, we obtained around 3152 colonies of KO riPS colonies corresponding to an efficiency rate of 1.26%.

Due to time restraints, only reprogramming with subsequent freezing of the picked colonies was possible.

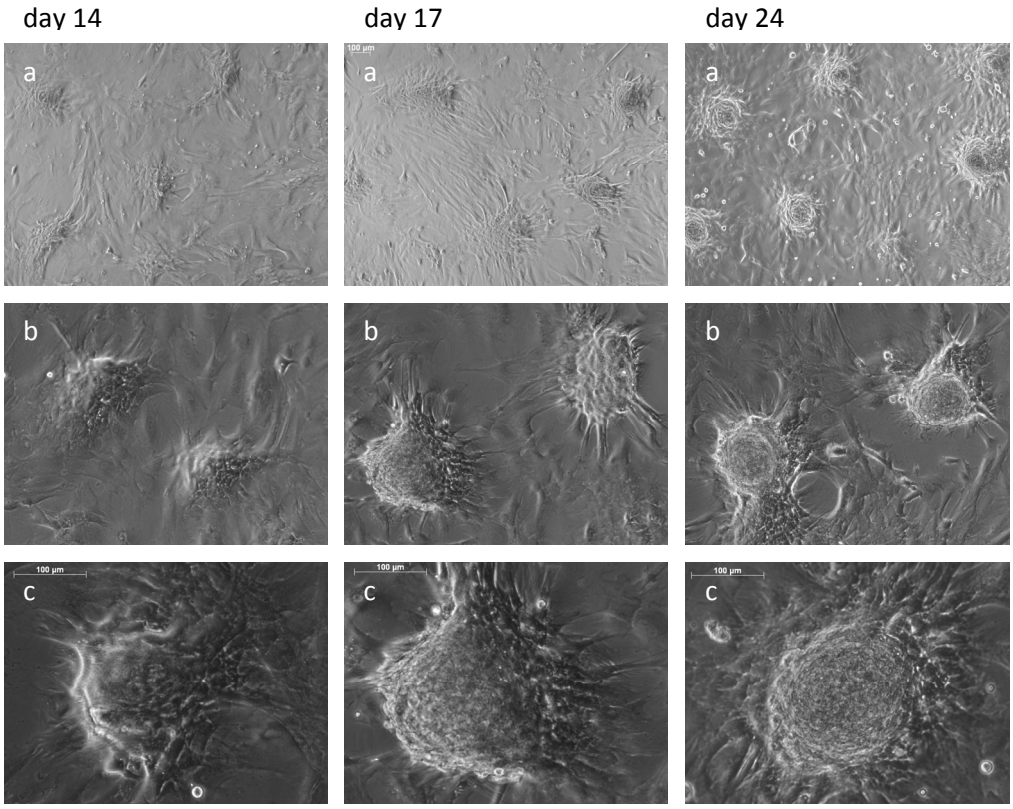

Fig. 22: Morphology of KO riPS-3 at different time points after infection with retroviruses. From the 12th day on, they formed round colonies, with shiny borders. At day 24, the day of picking, the colonies reached a very defined ES cell-like morphology. Magnification: (**a**) 5x (**b**) 10x (**c**) 20x

E. Discussion

The recent works on iPS cells open new dimensions in the study and characterization of pluripotent cells. Mouse iPS cells, generated by reprogramming somatic cells with defined transcription factors show typical characteristics of pluripotent stem cells (see Introduction), including their morphology, the expression of key markers of pluripotency, *in vitro* differentiation to derivates from all three germ layers and *in vivo* differentiation into teratomas. Moreover, these cells are able to generate germline chimeras (Okita et al., 2007; Takahashi and Yamanaka, 2006; Wernig et al., 2007).

Furthermore, four defined factors, Oct-3/4, Sox2, Nanog and Lin28 or Oct-3/4, Sox2, Klf4 and c-Myc, were sufficient to reprogram human somatic cells into pluripotent stem cells exhibiting the essential characteristics of embryonic stem cells (Takahashi et al., 2007; Yu et al., 2007).

The aim of this work was the generation and characterisation of riPS cells from REFs and the subsequent use of this technology to generate KO riPS cells out of KO rat fibroblasts established by Oliver Sterthaus in 2007 (see Introduction).

Reprogramming of rat embryonic fibroblasts using defined factors

Even though protocols for the generation of iPS cells from mouse and human fibroblasts were already published, no protocol was yet developed for the reprogramming of rat cells. We therefore decided to reprogram the REFs either with all four factors, Oct-3/4, Sox2, Klf4 and c-Myc or with the three following factors: Oct-3/4, Sox2 and Klf4. The reason for this was the possibility to reprogram human and mouse fibroblast in absence of c-Myc, and thereby eliminating the danger of inducing tumors in the chimeric animals generated with the iPS cells. The different retroviruses were produced in individual dishes containing Plat E cells instead of one dish for all retroviruses in order to increase the titer of the virus released in the medium. Afterwards the medium, containing four equal parts of each retrovirus-containing media, was used to infect the REFs plated on rat feeders (day 0). From day 2 on the infected REFs were cultivated in iPS medium.

Because of the absence of selection markers for the rat, the morphology parameter was solely used as selection criterion for picking the resulting riPS colonies. At the time of picking, both the riPS-4 and the riPS-3 colonies, formed tightly packed colonies with clear boundaries (see results, Fig. 1) similar to mouse ES cells. The reprogramming of rat fibroblast was only possible by combining rat feeders with addition of LIF in the medium. Mouse and human cells in contrast could be established by cultivation on SNL feeders producing LIF. Reprogramming of embryonic rat fibroblast on SNL feeders did not allow the formation of iPS cell colonies (data not shown).

Wernig. *et al*. (2008) demonstrated that the reprogramming process was substantially delayed in MEFs and the overall efficiency was reduced when c-Myc was not transduced: absence of c-Myc de-

layed the appearance of the colonies from six days with four factors to 30 days with only three factors. Our data indicated a marginal slower reprogramming process. The development of riPS-3 colonies was around three days delayed compared to riPS-4 colonies, but without a reduced efficiency rate, overall the reprogramming efficiency was low (< 0.05%).

For mouse and human iPS cells it was shown that chemical treatment with histone deacetylase inhibitors, e.g. VPA (see Introduction), and DNA methyltransferase inhibitors was able to improve the reprogramming efficiency of four factor-infected MEFs by more than 100-fold (Huangfu et al., 2008a). The use of these inhibitors or other chemical compounds for improving reprogramming efficiency of REFs has to be tested and established.

An important improvement would be the generation of riPS cells without using retroviruses or viral vectors allowing the generation of better riPS cells, especially when this technique would be applied to generate KO rats for biomedical research.

Summarized, the reprogramming process with REFs was overall similar to the one described for MEFs and human fibroblasts, with similar efficiencies but nevertheless needing the use of rat feeders and addition of LIF into the culture media. This process might be improved in several points, for example to increase the reprogramming efficiency and to produce riPS cells for research projects with less compunction regarding to tumor formation.

Cultivation of riPS cells

riPS cells turned out to be unstable in the first passages after picking. During cultivation, the morphology of both the riPS-4 and riPS-3 cells, changed their morphology. However, AP staining and IHC showed that the riPS colonies remained in a pluripotent state even when they had an uncharacteristic morphology. In later passages, some of the riPS cells migrated out of the colonies and arranged themselves around the enduring compact colonies (see Results, Fig. 3B + Fig. 5B). This behavior indicates a general instability of the cells in the cultivation media used.

After establishment and picking, the riPS cells were transferred on SNL feeders instead of rat feeders but the medium was still supplemented with LIF. The addition of LIF to the medium was essential for maintaining the cells in a pluripotent state.

It has been shown that the MEK signalling pathway and GSK3 are involved in the entry into differentiation of pluripotent stem cells (Chen et al., 2006; Frame and Cohen, 2001; Stavridis et al., 2007). Buehr *et al.* (2008) recently generated rat embryonic stem cells by using inhibitors of these two pathways. Moreover, these inhibitors tend to facilitate the generation of iPS cells from NS cells (Silva et al., 2008). Therefore, the riPS cells could benefit from these inhibitors, and be more stable in culture.

Characterisation analysis

We were interested in the generation of pluripotent riPS mainly because of their potential use in the generation of KO rats. For this reason we were interested in determining if these cells are able to be electroporated, a routine procedure for the generation of KO animals with ES cells. We therefore decided to electroporate the riPS-3 and riPS-4 cells with a vector encoding EGFP. Furthermore this genetic labelling would help to visualize the cells after blastocyst injection.

During cultivation the riPS cells were examined at different time points, especially for the presence of a correct set of chromosomes and of course for the expression of the characteristic markers for pluripotency.

The electroporation was successful in the riPS-4 and the riPS-3 cells and green fluorescent colonies (see Results, Fig. 6) were picked, expanded and analysed. The four factors cells were in general more difficult to electroporate; the reasons are not yet clear. A comparison between the riPS cells before and after electroporation was also performed and the results are discussed in the following sections.

Karyotype analysis

ES cells maintain a normal and stable karyotype during almost unlimited period of cultivation. In order to generate chimeric rats, the cells must have a stable and normal karyotype. It is therefore essential to evaluate the riPS cells karyotype.

The analysis showed that the riPS cells have a normal karyotype of 42 chromosomes with a telocentric and metacentric morphology (see Results, Fig. 7), before and after electroporation.

PCR analysis

A PCR analysis was performed to amplify the transduced mouse Oct-3/4, Sox2, Klf4 and c-Myc genes and to verify their presence in the riPS cells. The use of the pMXs vectors as a positive control indicated the right size of the expected bands.

In the riPS-4 clones the four factors were detected. However the quality of the c-Myc PCR was not optimal. Due to time restraints it was not possible to improve this PCR.

In the riPS-3 the three factors, Oct-3/4, Sox2 and Klf4, were amplified. We also performed a c-Myc PCR in the riPS-3 cells, which gave negative results. Therefore, no contamination with retroviruses containing the c-Myc transgene or riPS-4 factors had occurred.

In further PCRs, native REFs could be used as a negative control, as the pMX-S1811 primer which binds on the promoter pMX-S1811 of the pMXs-GW vector allows only the specific amplification of the viral vectors but not of the endogenous rat Oct-3/4, Sox2, Klf4 and c-Myc.

RT-PCR analysis by using transgene-specific primers would reveal the expression level of the transduced factors. As demonstrated in mouse iPS cells, the transgenes were largely silenced (Brambrink

et al., 2008; Stadtfeld et al., 2008a). This is an important analysis for further investigation of the generated riPS cells.

Alkaline phosphatase staining and immunohistochemical analyses

During cultivation, before and after electroporation, the riPS-4 and the riPS-3 cells were positive for the typical markers of pluripotency in stem cells, AP, Oct-3/4 and SSEA-1. In later passages, the cells which migrated out of the colonies were AP negative which leads to the assumption that these cells had begun to differentiate (see Results, Fig. 11A / riPS-4 not shown).

For the IHC analyses Oct-3/4 and SSEA-1 were used as pluripotency markers. Oct-3/4 is expressed in the nuclei of undifferentiated cells and SSEA-1 is expressed on the surface of undifferentiated cells.

IHC before electroporation

The Oct-3/4 staining is located in the center of each riPS-4 and riPS-3 cell suggesting a nuclear staining. The SSEA-1 staining is located at the cell surface.

In the analysis for the riPS-4 and riPS-3 cells Oct-3/4 positive cells appeared around the SSEA-1/Oct-3/4 stained colonies. We hypothesized that these single cells were the cells which migrate out of the colonies (described above) and were negative for the AP staining. Nevertheless, the cells located in the colonies in these passages were constantly AP positive.

The Oct-3/4 staining in these single cells was either due to the persistent expression of the transduced Oct-3/4 vector or to the fact that these cells still expressed the endogenous pluripotency marker Oct-3/4. The question is arising if these cells are still partially pluripotent cells and are still able to rebuild SSEA-1/Oct-3/4 positive colonies. FACS (fluorescence-activated cell sorting) provides a method for sorting cells based upon their specific fluorescent characteristics. It could be used to separate the red stained single cells (Oct-3/4 positive) from the red/green stained (Oct-3/4 and SSEA-1 positive) riPS-cells. The red stained single cells could then be analysed in respect to their behaviour in culture. This would reveal if these cells can still form appropriate colonies or if they immediately begun to completely differentiate and loose the Oct-3/4 staining.

IHC after electroporation

riPS-4 and riPS-3 cells still express both pluripotency markers Oct-3/4 and SSEA-1 after electroporation. The Oct-3/4 staining was located in the nuclei of each cell whereas the SSEA-1 staining was as expected on the surface of each cell.

The Oct-3/4 positive single cells, which were detected in IHC analyses before electroporation, were not seen in IHC after electroporation. A possible explanation is that these single cells could not be electroporated and subsequently died because they were not resistant to G418.

Summarized, the colonies, both riPS-4 and iPS-3 ones, expressed the typical pluripotency markers AP, Oct-3/4 and SSEA-1, before and after electroporation.

Differentiation potential *in vitro*

The riPS-4 and riPS-3 cells were able to form EB in suspension. When specifically induced (see Methods), the riPS-4 and riPS-3 cells were also capable to differentiate into specific cell types: smooth muscles, neurons and glial cells. Smooth muscles derive from the mesoderm, neurons and glial cells originate from the ectoderm.

These results suggest that the riPS cells should be able to differentiate also to specific cell types from the endoderm if an efficient protocol is available.

Differentiation potential *in vivo*

To test pluripotency *in vivo*, riPS cells were injected subcutaneously into NOD/SCID mice. Three weeks after injection we observed tumor formation for both riPS-4 and riPS-3 cells. Histological examination showed that the riPS-3 cells formed teratomas which consisted of all three germ layers, including cartilage structures (mesoderm), epithelial structures (ectoderm) and glandular structures (endoderm). Histological examination of the teratomas derived from riPS-4 cells has also to be done, to reveal the presence of tissues originating from all three germ layers.

We also injected riPS-3 cells expressing EGFP into blastocysts from Wistar RCC x Brown Norway. Three rounds of injections were performed. We transferred the embryos into six foster mothers and obtained one litter with eight newborns. However the newborns showed hardly any EGFP expression PCR analyses of hair from the resulted newborns confirmed the presence of the EGFP expressing vector and therefore the chimerism.

Summarised results for the riPS-4 and riPS-3 cells

Collectively our data demonstrates that the generated rat iPS cells show the expected properties of authentic pluripotent cells: a typical morphology, the expression of pluripotency markers AP, Oct-3/4 and SSEA-1, a stable karyotype, EB formation, induced differentiation to specific cell types, teratoma formation and the potential of riPS-3 to build chimeric rats. The generation of chimeric rats with riPS-4 cells and the potential of the riPS-4 and riPS-3 cells to colonize the germ line still has to be further examined.

Furthermore these results show that the pluripotent state of riPS-3 cells is equivalent to the pluripotency seen in riPS-4 cells. This is an important assessment in respect to the oncogenic character of c-Myc.

Our data are supported by the recent publication (January 9, 2009) of two papers demonstrating the generation of induced pluripotent stem cells from adult rat somatic cells (Li et al., 2009; Liao et al., 2009). Liao *et al.* (2009) transduced adult rat cells with lentiviral vectors expressing Oct-3/4, Sox2, Klf4 and c-Myc. The generated iPS cells expressed AP, SSEA-1, Oct-3/4 and other undifferentiated ES cells marker genes, showed a normal karyotype, were able to differentiate into three germ layers *in*

vitro and formed teratomas after an intramuscular injection. Li *et al.* (2009) reprogrammed rat liver progenitor cells with Oct-3/4, Sox2 and Klf4 and cultivated them in conventional mouse ES cell medium (containing knock out serum replacement and LIF) supplemented with a combination of MEK, GSK3 and FGF receptor inhibitors. The resulting iPS cells had typical characteristics of pluripotent cells. They had an ES cells morphology, expressed the right pluripotency markers, were able to differentiate *in vitro*, and formed teratoma *in vivo*. Most remarkably chimeric rats were born after blastocyst injection. However no germline transmission has been detected.

Their and our data may pave the way to generate gene-targeted rats out of riPS cells, once germline transmission is achieved.

In this context we reprogrammed KO rat fibroblasts, generated in our laboratory by Oliver Sterthaus (see Introduction) with the three factors Oct-3/4, Sox2 and Klf4. KO riPS-3 cells provide a possibility to generate KO rats.

Reprogramming of knock-out fibroblasts

The reprogramming efficiency rate amounted to 1.26% which is higher than the efficiency rate detected for riPS-4 and riPS-3 cells perhaps due to the hybrid background of these cells.

The generated KO riPS-3 cells grew in multilayered round colonies and exhibited clear boundaries. Both are typical characteristics of ES cell morphology.

Further investigation will show if the KO riPS-3 cell can be expanded. Furthermore several characterisation analyses will then be necessary to verify their pluripotent state, before injecting them into blastocysts to generate a KO rat.

Summary

The iPS technology provides a feasible approach to establish pluripotent stem cell lines for the rat, an animal in which ES cells have proven to be difficult to establish. In addition, riPS cells may offer the possibility to generate transgenic rats which could be used as model animals in biomedical research.

Furthermore the possibility to generate pluripotent stem cells following reprogramming of adult somatic cells represents a potential method to bypass the ethical debate surrounding ES cells. The destruction of an embryo or the nuclear transfer from somatic cell into an oocyte to collect ES cells would not be necessary anymore. Adult somatic cells would be sufficient to establish pluripotent stem cells. It would be possible to use a patient's own fibroblasts, to reprogram them into pluripotent cells, which could be used for therapeutical purposes.

However there are still several obstacles to overcome before (rat) iPS cells can be used routinely. Nevertheless, the research on reprogramming develops very fast, and advancements in this technique are published regularly.

Outlook

The riPS cells described above show several properties of authentic pluripotent stem cells. However some characterisation analyses were not performed because of time restraints. Therefore, it would be useful to perform the following experiments for the different cell types:

riPS-4 cells / riPS-3 cells:

- c-Myc PCR for riPS-4 cells: The PCR for c-Myc performed in this study was not optimal.
- Differentiation to endodermal cell types: The potential to differentiation to representative from the endoderm should be tested using an appropriate protocol.
- Karyotyping of the electroporated riPS-4 cells: The presence of a stable karyotype after electroporation should be examined.
- Differentiation potential of electroporated iPS-4 and iPS-3 cells: Their differentiation potential could be confirmed by EB formation and teratoma formation.
- Blastocyst injection with riPS-4 cells: The capacity of riPS-4 cells to participate in the development of an embryo should be tested.
- Repetition of the blastocyst injections for the riPS-3 cells: Blastocyst injections with riPS-3 cells should be repeated. The next injection should be performed using another clone, e.g. 3A2.

KO riPS-3 cells:

- Expansion of the picked KO riPS-3 clones: The expansion of the frozen KO riPS-3 clones has to be performed to check if these cells could be expanded in culture.
- PCR analysis for the expanded clones: The PCR analysis reveals the presence of the three factors in the KO riPS-3 cells.
- Southern Blot: Southern Blot should be performed to screen the expanded KO riPS-3 clones for the correct knock-out of the Neurotrypsin gene.
- Characterisation analyses of the correct clones: To confirm the pluripotent characteristic of the correct KO riPS-3 clones, the following experiments would be useful: karyotyping, AP staining, IHC for Oct-3/4 and SSEA-1, EB formation, differentiation *in vitro* and teratoma formation.
- Generation of chimeric KO rats: When the above mentioned characterisation analyses demonstrate the pluripotent state of the KO riPS-3 cells, they could be injected into blastocysts to generate a chimeric KO rat.

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Acknowledgements

I would like to express my sincere gratitude to Prof. K. Bürki for giving me the opportunity to carry out this work at the Institute for Laboratory Animal Science.

Many thanks to Dr. Paolo Cinelli for introducing me to this project. Thank you for your constant availability when I had questions and for your great support.

Elisa, thank you for supervising me, showing me all the skills necessary for my project, and always having time to answer my questions. It was a pleasure to spend time with you!

Igor, thank you for the amusing time we had together and for all your patience with my English. Note: they are round shaped and have shiny borders! ☺ “Es isch ä tolli Ziit gsi mit diär!”

Nicole, thanks for the great time we spend together!

Dagmar, ich danke Dir für die anregenden Unterhaltungen, welche wir über mehr oder weniger laborspezifische Themen geführt haben. ☺ Es war mir eine Ehre, Dich als „Sitznachbarin“ zu haben!

Zsuzsi, Deine aufgestellte und freundliche Art hat mich jeden Tag erfreut und ich danke Dir für die schöne Zeit!

Thanks to Olga for helping me with the differentiation experiments.

Reto, thanks for your help and tips.

Der grösste Dank gebührt meiner Familie: Meinen Eltern, für die Unterstützung während meiner gesamten bisherigen Studienzeit. Meinen Schwestern, für den mentalen Beistand - und Seraina zusätzlich für das Korrekturlesen meiner Arbeit.
Einen speziellen Dank an Ruedi, für seine graphische, gestalterische und geduldige Unterstützung während dieser Zeit.
Danke!!

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28.04.2009